BIANCA FACCHIM GONÇALVES

"CARCINOGÊNESE PROSTÁTICA QUIMICAMENTE INDUZIDA POR N-METIL N-NITROSURÉIA (MNU) EM GERBILOS DA MONGÓLIA: ASSOCIAÇÃO COM PROMOTORES ESTERÓIDES OU DIETA HIPERLIPÍDICA"

"PROSTATE CARCINOGENESIS CHEMICALLY INDUCED BY N-METHYL-N-NITROSOUREA (MNU) IN MONGOLIAN GERBILS: ASSOCIATION WITH STEROIDS PROMOTERS OR HIGH-FAT DIET"

Campinas, 2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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Orientador: Dr. Sebastião Roberto Taboga
Coorientadora: Dra. Silvana Gisele Pegorin de Campos

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Universidade Estadual de Campinas para obtenção do Título de Doutora em Biologia Celular e Estrutural, na área de Biologia Celular.

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Este exemplar corresponde à versão final da tese defendida pela aluna ***Bianca Facchim Gonçalves*** e orientada pelo Sebastião Roberto Taboga.

Assinatura do Orientador

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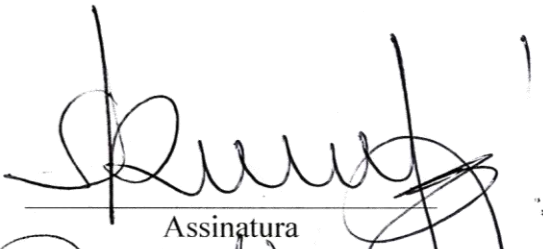

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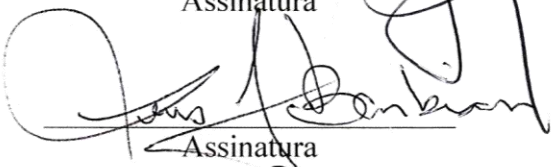

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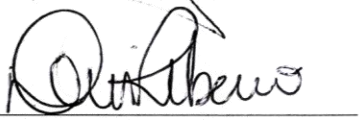

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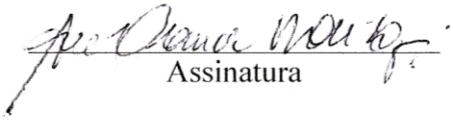

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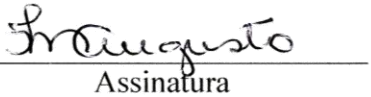

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RESUMO

Um dos principais desafios no campo de pesquisa do câncer prostático é a busca por sistemas modelo que permitam a investigação dos aspectos patológicos, bioquímicos e genéticos desta doença. O gerbilo *Meriones unguiculatus* tem possibilitado a avaliação de lesões prostáticas e sua evolução de estágio benigno para maligno (invasivo) após período relativamente curto de tratamento com o carcinógeno N-Metil-N-Nitrosuréia (MNU), um potente causador de metilação do DNA por ação direta. Assim, o presente trabalho teve por objetivos: 1) Determinar a incidência, multiplicidade e latência tumoral de lesões espontâneas e quimicamente induzidas nos lobos prostáticos ventral e dorsolateral do gerbilo; 2) Investigar se o estradiol exerce papel protetor e/ou promotor sobre neoplasias induzidas por MNU; 3) Avaliar o potencial promotor da dieta hiperlipídica sobre a carcinogênese induzida na próstata ventral; 4) Analisar a participação de produtos alterados de genes *ras* e do *status* global de metilação do DNA do epitélio prostático no processo tumoral mediado por MNU. Para tanto foram utilizados animais adultos submetidos à dose única intraperitoneal de MNU (50mg/Kg), exceto o grupo controle. Os grupos experimentais foram submetidos à exposição crônica de andrógeno, estradiol ou dieta hiperlipídica por 14 e 28 semanas. As metodologias aplicadas envolveram análises quantitativas e estatísticas de multiplicidade e incidência de lesões prostáticas, peso corporal, acúmulo de gordura corporal, peso prostático, dosagens hormonais, índice proliferativo, cariometria, frequência de células AR-positivas e basais, *status* global de metilação e determinação da expressão de proteínas. O modelo de indução tumoral prostática por MNU associado à testosterona no gerbilo se mostrou eficaz, pois reduziu a latência tumoral e permitiu o estudo de estágios avançados da carcinogênese após curto período. As neoplasias se manifestam inicialmente no lobo dorsolateral e requerem um tempo maior para se estabelecer no lobo ventral. No entanto, a progressão de lesões pré-malignas para malignas ocorre de maneira mais significativa no lobo ventral. Isso indica que a progressão tumoral ocorre de maneira distinta entre os lobos prostáticos e que vias alternativas estão possivelmente envolvidas nesse processo. A longa exposição a altas doses de estrógeno foi capaz de prevenir e reduzir a taxa de crescimento tumoral. Apesar dos efeitos terapêuticos contra a progressão neoplásica, a terapia estrogênica levou ao estabelecimento de um epitélio com características distintas da próstata normal, como: mudanças no padrão de metilação do

DNA e aumento de células basais e AR positivas. Juntos, esses eventos contribuíram para criar um ambiente epitelial instável que pode provocar a recidiva das lesões em períodos subsequentes. A associação entre MNU e dieta hiperlipídica promoveu aumento na incidência de lesões estimuladas pelo carcinógeno isoladamente, as quais apresentaram maior número de células AR-positivas, ruptura da camada de músculo liso indicando microinvasão tumoral, e alta reatividade para metaloproteinase do tipo 2. A análise molecular indicou alta expressão das proteínas Ras em tecidos induzidos por MNU, sugerindo a participação dessa via na promoção e progressão de tumores prostáticos. Assim, conclui-se que a dieta hiperlipídica pode ser considerada um agente promotor da carcinogênese prostática, e o gerbilo representa um bom modelo para estudos histopatológicos.

ABSTRACT

One of the major challenges in the field of prostate cancer research is the search for model systems that allow the investigation of pathological, biochemical and genetic factors of this disease. The gerbil *Meriones unguiculatus* has enabled the evaluation of prostate lesions and evolution from benign to malignant (invasive) stage after a relatively short period of treatment with the carcinogen N-methyl-N-nitrosourea (MNU), a potent causative of DNA methylation by direct action. Thus, this study aimed to: 1) Determine the incidence, multiplicity, and tumor latency of spontaneous and chemically-induced lesions in ventral and dorsolateral gerbils' prostatic lobes; 2) Investigate whether estradiol exerts protective and/or promoter hole on neoplasms induced by MNU; 3) Evaluate the promotional potential of high-fat diet on induced-carcinogenesis in ventral prostate; 4) Analyze the involvement of altered *ras* gene products and the global DNA methylation status of prostate epithelium on MNU-mediated tumor process. Therefore, we used adult animals subjected to a single intraperitoneal dose (50mg/kg) of MNU, except the control group. The experimental groups were subjected to chronic exposure of androgen, estradiol or high-fat diet for 14 and 28 weeks. The methodologies involved quantitative and statistical analysis of multiplicity and incidence of prostatic lesions, body weight, body fat accumulation, prostate weight, hormonal measurements, proliferative index, karyometry, frequency of AR-positive and basal cells, global methylation status and determination of protein expression. The model of prostatic tumor induction by MNU associated with testosterone in the gerbil was effective because it reduced tumor latency and allows the study of advanced stages of carcinogenesis after short period. Neoplasms manifest initially in dorsolateral lobe and require a longer time to be established in ventral lobe. However, the progression from premalignant to malignant lesions occurs more significantly in the ventral lobe. This indicates that tumor progression occurs differently between prostatic lobes and alternative pathways maybe possibly involved in this process. Long exposure to high doses of estrogen was able to prevent and reduce the rate of tumor growth. Despite therapeutic effects against neoplastic progression, estrogen therapy led to the establishment of an epithelium with distinct characteristics from normal prostate, such as changes in the pattern of DNA methylation and increased amount of basal cell and AR-positive cells. Together, these events contributed to create an unstable epithelial compartment

that can cause lesions recurrence in subsequent periods. The association between MNU and high-fat diet promoted an increase in incidence of lesions stimulated by carcinogen alone, which had a higher number of AR-positive cells, disruption of the smooth muscle layer indicating tumor microinvasion and high reactivity for metalloproteinase type 2. Molecular analysis indicated high expression of Ras proteins in tissues induced by MNU, suggesting the involvement of this pathway in the promotion and progression of prostate tumors. Thus, we conclude that the high-fat diet can be considered a promotional agent of prostate carcinogenesis and that gerbil is a good model for histopathological studies.

SUMÁRIO

"Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes".

Martin Luther King

Dedico

Aos meus pais, Élide e Luiz

Minha origem, meu amor, meus maiores exemplos

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LISTA DE ABREVIATURAS

5mC – 5-Metilcitidina

AR – Do inglês *Androgen Receptor*

C – Grupo controle

CRPC – Do inglês *Cancer Resistant Prostate Cancer*

DAB – 3-3`-diaminobenzidina tetrahidrocloreto

DL – Lobo dorsolateral prostático, do inglês *Dorsolateral Lobe*

ER – Do inglês *Estrogen Receptor*

ML – Músculo liso

MMP-2 – Metaloproteinase-2

MNU – N-metil-N-nitrosuréia

MNU+D – N-metil-N-nitrosuréia associado à dieta hiperlipídica

MNU+E – N-metil-N-nitrosuréia associado à estradiol

MNU+T – N-metil-N-nitrosuréia associado à testosterona

NIP – Neoplasia Intraepitelial Prostática

PCNA – Do inglês *Proliferating Cell Nuclear Antigen*

PIN – Do inglês *Prostatic Intraepithelial Neoplasia*

PSA – Do inglês *Prostatic Specific Antigen*

VL – Lobo ventral prostático, do inglês *Ventral Lobe*

INTRODUÇÃO

Morfofisiologia da Próstata

A próstata é uma glândula acessória do sistema genital masculino que juntamente com a glândula seminal contribui com a produção de nutrientes para o fluido seminal e promove a manutenção do gradiente iônico e pH adequados desta secreção (Untergasser *et al.,* 2005). Grande importância científica tem sido atribuída a este órgão uma vez que, o câncer prostático é o tumor maligno mais prevalente em homens de países ocidentais industrializados (Goering *et al.*, 2012). A patologia molecular desta doença é complexa, pois além de ser altamente relacionada à idade, fatores hereditários e andrógeno-dependentes, é também influenciada por hormônios esteróides sexuais endógenos, fatores ambientais, dietas, respostas imunes e inflamatórias (Huynh *et al.,* 2001; Carruba, 2006; De Marzo *et al.,* 2007).

A morfogênese prostática tem início a partir do seio urogenital, durante o período fetal, e é dependente de andrógenos circulantes produzidos pelos testículos fetais (Cunha *et al.,* 1986; Thomson & Cunha, 1999; Thomson *et al.,* 2002). O crescimento e desenvolvimento da glândula se estendem até que a maturidade sexual seja atingida e a manutenção de sua homeostase no indivíduo adulto é garantida por hormônios esteróides como a testosterona (Cunha *et al.,* 1986; Marker *et al.,* 2003). Os efeitos androgênicos em células alvo resultam da interação do hormônio com receptores de andrógenos (AR) presentes nestas (Cunha *et al.,* 1986). A produção de andrógenos é regulada pelo eixo hipotalâmico-hipofisário-gonadal (Debes e Tindall, 2002), com mais de 95% da testosterona produzida pelas células de Leydig dos testículos e menos de 5% pelas glândulas adrenais (Hsing *et al.,* 2002).

O desenvolvimento e crescimento da próstata de roedores e de humanos ocorrem de maneira análoga (Cunha *et al.,* 2004a,b). Em ratos e camundongos a próstata é uma glândula túbulo-alveolar composta, formada por um complexo sistema de ductos que partem da uretra e terminam distalmente em ramos. Nesses roedores é composta por quatro lobos distintos: anterior (também chamado de glândula coaguladora), dorsal, lateral e ventral (Figura 1A), os quais se arranjam circunferencialmente ao redor da bexiga e apresentam características particulares de ramificação de ductos e produção de secreções proteicas (Sugimura *et al.,* 1986; Hayashi *et al.*, 1991). Em um mesmo lobo, os ductos prostáticos apresentam heterogeneidade

regional quanto ao tipo celular, resposta a andrógenos, síntese e secreção de proteínas (Banerjee *et al.,* 1998).

De modo semelhante aos roedores, nos humanos a próstata também consiste de uma estrutura túbulo-alveolar, composta de ductos e alvéolos, com atividade secretora ligada principalmente à porção alveolar, embora os ductos também secretem substâncias que compõem o fluido de secreção prostático (Reese *et al.*, 1986). Entretanto em humanos, a próstata tem uma morfologia mais compacta, sem lobos distintos, sendo geralmente diferenciada em três zonas: central, de transição e periférica (McNeal, 1983). Em termos de homologia entre espécies, nota-se que o lobo ventral da próstata do rato corresponde à zona de transição da próstata humana e o lobo dorsal à zona periférica da próstata humana (Figura 1B) (Berquin *et al.,* 2005).

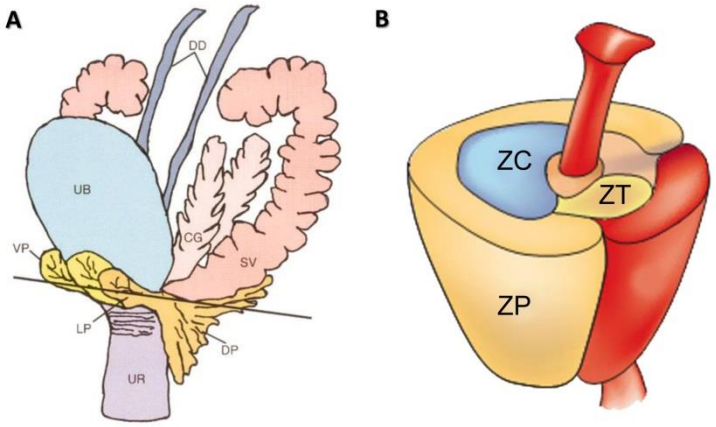


FIGURA 1. Representação esquemática da próstata de roedores (A) e humana (B). **A)** Vista anterolateral em roedores. VP – Próstata Ventral, LP – Próstata Lateral, DP – Próstata Dorsal, SV – Vesícula Seminal, CG – Glândula Coaguladora ou Próstata Anterior, DD – Ducto Deferente, UB – Bexiga Urinária. **B)** Visão interna da próstata humana. ZC – Zona Central, ZP – Zona Periférica, ZT – Zona de Transição (Adaptado de Shappell *et al.,* 2004 e De Marzo *et al.,* 2007).

O epitélio prostático é composto de quatro tipos de células: basal, secretora, intermediária e neuroendócrina que são dependentes dos hormônios esteróides e reagem diferentemente a cada um deles (De Marzo *et al.*, 1998; Rumpold *et al.*, 2002). Na próstata adulta as células secretoras luminais e intermediárias são os tipos mais frequentes, sendo responsáveis pela síntese e secreção, no lúmen alveolar, de diversas proteínas que irão compor o ejaculado masculino, incluindo antígeno prostático específico (PSA) e fosfatases específicas. Menos numerosas, as células basais estão geralmente restritas ao compartimento basal do

epitélio onde atuam como fonte progenitora das células secretoras. Na próstata humana as células basais formam uma camada contínua entre o epitélio secretor e a lâmina basal, o que não é observado em roedores e outras espécies, onde as poucas células basais aparecem em camadas descontínuas ao redor dos ductos. O epitélio prostático contém ainda células neuroendócrinas e algumas células do sistema imune, como macrófagos e linfócitos (McNeal, 1997; Chatterjee, 2003). Os tipos de populações celulares são similares entre as espécies e, provavelmente, desempenham as mesmas funções fisiológicas, porém a distribuição relativa dessas populações varia (Imamov *et al.,* 2004).

A camada epitelial e o componente estromal são separados por uma lâmina basal altamente organizada e composta principalmente por colágeno tipo IV e laminina. Entremeando as porções glandulares há um estroma conjuntivo ricamente vascularizado, com poucas fibras colágenas e elásticas além de células musculares lisas dispostas concentricamente aos alvéolos (Carvalho e Line, 1996). As células musculares lisas exercem papel contráctil durante a ejaculação e juntamente com os fibroblastos são responsáveis pela síntese dos componentes da matriz extracelular. Entre os elementos estruturais que integram a matriz destacam-se as fibras de colágeno, reticulares e elásticas, as quais conferem resistência mecânica e flexibilidade ao tecido, servindo como substrato para a ancoragem e migração celular (Tuxhorn *et al.*, 2001; Vilamaior *et al.*, 2005).

Os componentes glandulares de natureza epitelial e os não glandulares, de natureza estromal, apresentam características comuns nos diferentes animais independendo do aspecto macroscópico da glândula, o que favorece estudos referentes à homologia morfofuncional entre diferentes espécies (Price, 1963; Karr *et al.*, 1995).

O gerbilo como modelo para o estudo da biologia prostática

A utilização de gerbilos (*Meriones unguiculatus*) (Figura 2), roedores murídeos da subfamília Gerbillinae, é cada vez maior na pesquisa científica das áreas da imunologia (Jeffers *et al.,* 1984; Nawa *et al.,* 1994), fisiologia (Nolan *et al.,* 1990), culturas de células (Moritomo *et al.,* 1991) e morfologia (Redecker, 1991; Aoki & Komori *et al.,* 1994; Jones *et al.,* 1997; Santos *et al.,* 2000; Santos e Taboga, 2002; Corradi *et al.,* 2004; Rochel *et al.,* 2007; Fochi *et al.,* 2008; Scarano

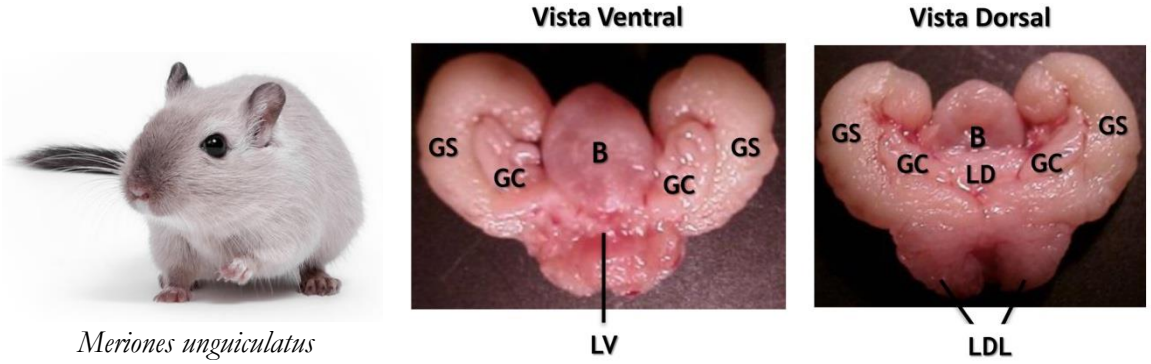
et al., 2008; Pinto *et al.,* 2010; Campos *et al.,* 2010; Gonçalves *et al.,* 2010). Este roedor tem se revelado um bom modelo para o estudo da próstata apresentando respostas significativas quanto a tratamentos hormonais (Santos *et al.*, 2006; Scarano *et al.*, 2006; 2008), drogas contra hiperplasia prostática humana (Corradi *et al.*, 2004), carcinogênese química (Gonçalves *et al.,* 2010; 2013) bem como, desenvolvimento de neoplasias espontâneas associadas ao envelhecimento (Pegorin de Campos *et al.*, 2006; Campos *et al.,* 2008; Custódio *et al.,* 2008; Campos *et al.,* 2010; Campos *et al.,* 2011).

De anatomia similar à do rato e camundongo, os gerbilos adultos de ambos os sexos variam entre 11,5 e 14,5 cm de comprimento corporal, sendo que os machos nesta faixa etária pesam em torno de 70 gramas. Este roedor representa um bom modelo experimental no sentido de tolerar distintas condições experimentais, mantendo-se saudáveis mesmo após longos períodos em biotério.

A glândula prostática do gerbilo é anatômica e histologicamente similar a dos demais roedores (Figura 2). Apesar de apresentarem similaridades com relação à morfologia e estrutura, os lobos prostáticos do gerbilo diferem quanto à ultraestrutura das células secretoras, mecanismos de secreção do fluido prostático e componentes da matriz extracelular (Rochel *et al.*, 2007). Dentre eles, o lobo ventral tem sido o mais estudado por nosso grupo de pesquisa devido as semelhanças com a próstata humana e por ser o mais responsivo às alterações hormonais (Pegorin de Campos *et al.*, 2006; Cordeiro *et al.*, 2008). Entre as porções glandulares, há um estroma conjuntivo vascularizado, com poucas fibras conjuntivas e elásticas, além de células musculares lisas bem compactadas, dispostas ao redor de cada alvéolo (Rochel *et al.,* 2007). Estas características teciduais e a disposição dos elementos epiteliais, estromais e musculares são também muito semelhantes às encontradas na próstata humana. Desta forma, as semelhanças morfofuncionais estabelecidas até o momento favorecem situações experimentais e correlações com o homem.

Trabalhos anteriores do nosso grupo tem sugerido uma predisposição para o desenvolvimento de neoplasias prostáticas espontâneas em gerbilos adultos, as quais se tornam mais frequentes com o envelhecimento (Pegorin de Campos *et al.*, 2006; Campos *et al.,* 2008). Partindo destas evidências foram conduzidos os primeiros trabalhos do grupo associando carcinógeno químico e andrógeno visando acelerar o processo de carcinogênese na glândula (Zanetoni, 2007; Gonçalves *et al.*, 2010). Os resultados foram promissores e após 3 meses de

tratamento, gerbilos adultos apresentaram incidência elevada de alterações histopatológicas que à semelhança de outros roedores e humanos, se caracterizaram por NIP, carcinomas microinvasivos e adenocarcinomas. No entanto, os trabalhos focaram principalmente as alterações estromais decorrentes das lesões no lobo ventral prostático (Zanetoni, 2007; Gonçalves *et al.*, 2010). Desta forma, uma investigação mais acurada do processo tumoral bem como da eficiência da indução da carcinogênese na próstata deste roedor podem fornecer as bases para o estabelecimento de um modelo alternativo para estudo da doença.



Meriones unguiculatus

FIGURA 2. O gerbilo da Mongólia, seu complexo prostático e glândulas associadas. GS – Glândula Seminal, GC – Lobo anterior ou Glândula Coaguladora, B – Bexiga Urinária, VL – Lobo Ventral, LD – Lobo Dorsal, LDL – Lobo Dorsolateral (Adaptado de Rochel *et al.,* 2007).

Carcinogênese Prostática

A última estimativa mundial apontou o câncer da próstata como sendo o segundo tipo de câncer mais frequente em homens e o sexto tipo mais comum no mundo, representando cerca de 10% do total de cânceres. Aproximadamente 75% dos casos diagnosticados no mundo ocorrem em países desenvolvidos. Esta patologia é considerada o câncer da terceira idade, uma vez que os maiores índices ocorrem a partir dos 65 anos (INCA, 2012).

Assim como outros canceres o câncer de próstata se desenvolve através do acúmulo de mutações somáticas e alterações epigenéticas que resultam em inativação de genes supressores tumorais e ativação de oncogenes (De Marzo *et al.*, 2007). Os genes afetados são mutados de uma maneira que subverte sua função normal, contribuindo assim para os vários processos considerados característicos do câncer como a autossuficiência em sinais de crescimento, insensibilidade aos sinais anti-crescimento, evasão de apoptose, aquisição de potencial

replicativo ilimitado, angiogênese sustentada, invasão tecidual e metástase (Hanahan & Weinberg, 2000; Umar *et al.*, 2012).

A aquisição dessas propriedades ocorre em três fases distintas que caracterizam o processo de carcinogênese: iniciação, promoção e progressão. A iniciação é a primeira fase do processo e consiste na aquisição de mutações, o que geralmente é induzido por um agente mutagênico. A promoção corresponde à etapa em que as células geneticamente alteradas sofrem os efeitos de agentes que estimulam a atividade mitótica, o que acelera a expansão clonal de uma célula iniciada e aumenta a probabilidade de outros eventos mutagênicos dando sequência ao processo de carcinogênese. A progressão por sua vez consiste na aquisição de fenotípo e comportamento agressivos pelas células tumorais, as quais passam a apresentar propriedades invasivas e potencial metastático (Withrow & Vail, 2007). O tempo entre o início até a aquisição de fenótipo invasivo é específico para diferentes órgãos e tecidos e dependente do tempo que cada tipo de câncer leva para o acúmulo das alterações genéticas (Umar *et al.*, 2012). No câncer prostático humano, por exemplo, o tempo estimado para o inicio do processo é de mais de 15 anos e neste estágio a doença é geralmente confinada ao órgão (Johansson *et al.,* 1997).

Clinicamente, os tumores são classificados como apresentando diferentes "graus", que correspondem a um conjunto de marcadores fisiológicos e histológicos (tais como a perda de diferenciação, ploidia anormal e morfologia) que se correlacionam com o perfil do paciente (Pedraza-Fariña, 2006). Histologicamente o câncer de próstata se desenvolve através de etapas bem definidas, a partir de Neoplasia Intraepitelial Prostática (NIP, considerada como lesão pré-maligna), para carcinoma localmente invasivo (*in situ*), e finalmente para o estágio metastático (Figura 3) (Rouet *et al.* 2010). A neoplasia intraepitelial tem sido aceita como uma lesão precursora do câncer de próstata (Bostwick *et al.*, 2000). Histologicamente esta consiste na aglomeração e estratificação de células com formato atípico, havendo variações no tamanho e forma nucleares, hipercromasia e presença de nucléolos evidentes. No entanto, a NIP fica retida no limite alveolar e a lâmina basal bem como a camada de células basais são mantidas (Brawer, 1992). Com a progressão tumoral e consequente aquisição de fenótipos malignos ocorre a invasão do estroma adjacente por células neoplásicas. Em função das relações estruturais, a ruptura da camada de células basais e da lâmina basal são pré-requisitos para a invasão do tumor prostático (Liu *et al.*, 2009). Inicialmente pode ocorrer a invasão local do

tecido glandular por pequenos grupos de células (Carcinoma microinvasivo), posteriormente o foco invasivo pode atingir proporções maiores envolvendo formação glandular inequívoca, caracterizando os adenocarcinomas (Shappel *et al.*, 2004). No adenocarcinoma prostático uma diversidade de graus é atingida, sendo uma doença heterogênea não somente pelos seus graus, mas também pela genética, ploidia e expressão de oncogêneses (Lucia *et al.*, 1998). Assim, uma célula pode passar por diversos fenótipos durante o processo de tumorigênese até atingir o fenótipo maligno.

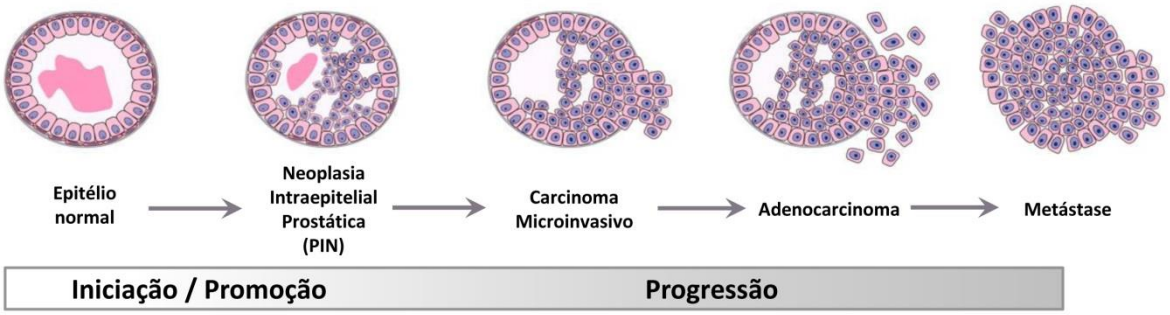


FIGURA 3. Fases do processo tumoral na próstata evidenciando as alterações citológicas e na histoarquitetura dos alvéolos durante a progressão neoplásica (Adaptado de Shen & Abate-Shen, 2010).

Modelos Experimentais para estudo do Câncer de Próstata

Um dos principais obstáculos para o desenvolvimento de estratégias terapêuticas para o câncer prostático tem sido a falta de modelos animais adequados que possibilitem a avaliação destas estratégias. Vários grupos vêm conduzindo pesquisas com espécies de murinos com o objetivo de estabelecer aquele que melhor represente os aspectos patológicos e bioquímicos do câncer prostático humano (McCormick *et al.,*1998; Shirai *et al.*, 2000; Huss *et al.*, 2001).

A combinação do carcinógeno N-metil-N-nitrosuréia (MNU) e andrógeno tem emergido como uma boa ferramenta para estudos envolvendo a carcinogênese prostática induzida quimicamente em diversas espécies e linhagens de roedores, visto que promove alta incidência de lesões proliferativas como Hiperplasia, Displasia e NIP já no início do tratamento (Bosland *et al.*, 1983; Shirai *et al.*, 2000; Pollard e Luckert, 1987; 1992; Liao *et al.*, 2002; 2005; Boileau *et al.*, 2003; Arunkumar *et al.*, 2006). MNU é um agente metilante de ação direta (Figura 4) que reage com macromoléculas celulares incluindo proteínas e DNA

(Beranek, 1990; Jiricny, 2006; van Zeeland *et al.*, 2008). Sua administração leva a formação de grandes quantidades de O^6-metilguanina, um aducto de DNA com propriedades citotóxicas e recombinantes (Kaina *et al.*, 2007) o que o relaciona com o aumento do risco de desenvolvimento de câncer (Kyrtopoulos, 1995). Desta forma a administração de MNU pode afetar uma variedade de órgãos nos quais o desenvolvimento de neoplasias é influenciado por agentes promotores, por exemplo, tumores de mama e intestino influenciados por dietas ricas em gordura insaturada (Chang *et al.,* 1977) e tumores de bexiga associados a metil metanosulfonato (Tudor *et al.,* 1984). Dessa forma, parece comum que MNU sensibilize muitos órgãos e que o tipo e sítio tumoral desenvolvido por cada animal seja determinado pelo agente promotor e possivelmente pela linhagem em investigação (Pollard e Luckert, 1986; Sukumar *et al.*, 1991).

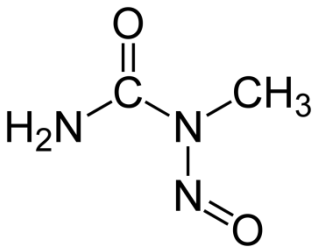


FIGURA 4. Estrutura química do carcinógeno N-metil-N-nitrosuréia.

Poucos estudos têm caracterizado biomarcadores neste modelo, no entanto a inter-relação entre angiogênese, proliferação e apoptose, durante a carcinogênese prostática induzida por MNU mostra uma forte correlação com dados humanos publicados (Liao *et al.*, 2002). Para Bosland e Prinsen (1990) a administração de andrógeno está associada a um aumento decisivo da proliferação celular durante a fase inicial da carcinogênese química prostática. Portanto, as doses de testosterona são administradas para estimular a proliferação de células epiteliais e maximizar a sensibilidade prostática ao insulto carcinogênico (McCormick *et al.,* 1998). Em geral, este insulto conduz a vias de sinalização proliferativas ativadas irreversivelmente, as quais se tornam independentes de hormônios promotores de crescimento, fatores de crescimento e citocinas (Liao *et al.,* 2002). Em ratos da linhagem Wistar-Unilever o carcinógeno causa o aparecimento de lesões proliferativas nos lobos anterior e dorsolateral. Esses lobos, em ratos, são considerados homólogos às áreas de maior susceptibilidade a tumores da próstata humana (Boileau *et al.*, 2003).

Embora o câncer prostático seja o segundo tipo mais comum de câncer e a terceira causa mais frequente de morte no mundo ocidental, sua patogênese é relativamente pouco compreendida ao nível genético (Konishi *et al.,* 1995). Membros da família de proto-oncogenes *ras* são os genes mais comumente detectados em tumores humanos e de animais induzidos por carcinógeno e podem desempenhar uma função causal no desenvolvimento destes tumores (Bos, 1989). Esses genes codificam um grupo de proteínas-G heterotriméricas, que após modificações pós-traducionais são translocadas do citosol para a membrana plasmática. O produto normal do gene H-*ras*, por exemplo, está envolvido na proliferação e morte celular, portanto possui um efeito anti-tumorigênico quando não-mutado (Lowy e Willumsen, 1993).

Aproximadamente 75% dos carcinomas prostáticos induzidos em ratos por MNU e subsequente tratamento crônico com testosterona carregam uma mutação GGA – GAA na segunda posição do códon 12 do proto-oncogene Ki-*ras* ou H-*ras* (Sohemy e Archer, 2000; Imaoka *et al.,* 2005). Estes achados sugerem que a via de carcinogênese prostática que envolve genes *ras* requer a ativação destes nos componentes epitelial e estromal da glândula (Condon *et al.,* 1999).

Um outro modelo que promove alta incidência de carcinogênese prostática é o que envolve a administração crônica de estrógeno em roedores (Bosland, 1992; Fujimoto *et al.,* 2004). No epitélio as alterações envolvem metaplasia escamosa e no estroma, proliferação de fibroblastos e possível desdiferenciação de células musculares lisas (Bianco *et al.,* 2002). A expressão alterada de receptor de estrógeno (ER) pode ocorrer em câncer de próstata sugerindo um envolvimento desse receptor na progressão de lesão proliferativa (Royuela *et al.,* 2001).

Os resultados provenientes da associação entre carcinógenos químicos como o MNU e estrógeno são divergentes. Shirai *et al.* (1987) não constataram efeito na promoção de tumores após administração deste esteroide e, segundo Pollard *et al.* (1989), o estrógeno pode exercer um efeito benéfico em estágios iniciais da tumorigênese prostática, diminuindo a incidência de adenocarcinomas. Esses trabalhos, porém não podem ser tomados como conclusivos da não associação entre progressão tumoral e tratamento com MNU + estrógeno, uma vez que a resposta prostática a estes esteróides depende de variáveis adicionais como: dosagem, tempo de exposição e presença de andrógenos (Bianco *et al.,* 2002). A retomada de estudos associando

esses dois elementos pode trazer novas perspectivas a respeito de vias patogênicas ou quimioprevenção para o câncer prostático.

Estudos epidemiológicos têm mostrado a associação entre uma dieta rica em lipídios e alta taxa de mortalidade por câncer de mama, cólon e próstata em humanos. Além disso, o consumo de dietas de alto valor calórico se relaciona com o crescimento de tumores prostáticos em roedores (Mukherjee *et al.*, 1999). Níveis hormonais de ratos ACI/Seg que consumiram 5 ou 20% de óleo de milho, correspondendo respectivamente à dieta com baixo e alto teor lipídico, durante longos períodos, indicaram que a testosterona aumentou significativamente e pode ter favorecido o desenvolvimento de neoplasias (Kondo *et al.,* 1994). Entretanto, o efeito promotor de uma dieta rica em lipídios sobre a carcinogênese experimental não tem sido bem estabelecido para a próstata.

O consumo de dietas ricas em gordura saturada por longos períodos levou ao aumento do peso do lobo ventral prostático em ratos (Cai *et al.,* 2001; Cai *et al.,* 2005; Escobar *et al.,* 2009). Gerbilos adultos que receberam óleo de milho semanalmente desenvolveram lesões proliferativas quando este foi administrado isolado ou em associação com MNU, indicando um possível papel promotor dessa dieta sobre o microambiente prostático (Gonçalves *et al.,* 2010). Os últimos dados sugerem que esse modelo roedor pode trazer melhores respostas sobre vias de promoção de carcinogênese associando MNU e dieta rica em lipídeos.

A aquisição de alta incidência de lesões prostáticas em curto período de tempo (6 meses) após administração de dose única de MNU seria suficiente para justificar sua importância na ampliação de conhecimentos a respeito da histogênese e evolução do câncer prostático. As diversas possibilidades de combinações entre o carcinógeno e outros potenciais promotores do câncer como esteróides e dieta rica em lipídeos, também trazem formas de compreensão de diferentes vias moleculares que participam conjuntamente ou não do estabelecimento de sítios de células anômalas. Adicionalmente cada uma das alterações impostas pelo modelo de carcinogênese mediada por MNU, mimetiza achados de estudos envolvendo tecidos prostáticos humanos e apoiam ainda mais a importância deste sistema para elucidar mecanismos da carcinogênese relevantes para a doença humana e sua prevenção.

OBJETIVOS

Objetivo Geral:

Caracterizar o modelo de carcinogênese prostática induzida por N-metil-N-nitrosuréia (MNU) na próstata de gerbilos adultos por meio da avaliação de biomarcadores epiteliais e estromais em lesões proliferativas.

Objetivos Específicos:

- Determinar a incidência, multiplicidade e latência tumoral de lesões espontâneas e quimicamente induzidas nos lobos ventral e dorsolateral da próstata do gerbilo;
- Investigar o papel protetor e/ou promotor do estradiol sobre neoplasias induzidas por MNU e avaliar por métodos imunohistoquímicos o epitélio prostático após prolongada terapia;
- Avaliar o potencial promotor da dieta hiperlipídica sobre a carcinogênese induzida por MNU na próstata ventral do gerbilo;
- Analisar a possível participação de produtos alterados de genes da família de proto-oncogenes *ras* (H-*ras*, K-*ras*, N-*ras*) e do *status* global de metilação do DNA do epitélio prostático no processo tumoral mediado por MNU.

RESULTADOS

Durante a execução deste trabalho foram empregadas metodologias específicas, como: análise histopatológica, imunohistoquímica, reconstrução tridimensional e análise molecular de expressão de proteínas. Os experimentos foram conduzidos em laboratórios de duas instituições de ensino:

- **Laboratório de Microscopia e Microanálises** – Ibilce/UNESP, São José do Rio Preto, SP – Brasil (Experimentação animal, Análise histológica, Imunohistoquímica e Reconstrução tridimensional);

- **Departamento de Clínica Veterinária** da Faculdade de Medicina Veterinária e Zootecnia da UNESP de Botucatu, SP – Brasil (Análise histopatológica);

- **Laboratório de Desreguladores Endócrinos e Carcinogênese Experimental** – IB/UNESP, Botucatu, SP – Brasil (Análise molecular de expressão de proteínas).

Os dados resultantes deste trabalho foram reunidos em três artigos que se seguem.

ARTIGO I

A NEW PROPOSED RODENT MODEL OF CHEMICALLY-INDUCED PROSTATE CARCINOGENESIS: DISTINCT TIME-COURSE PROSTATE CANCER PROGRESSION IN THE DORSOLATERAL AND VENTRAL LOBES

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A NEW PROPOSED RODENT MODEL OF CHEMICALLY-INDUCED PROSTATE CARCINOGENESIS: DISTINCT TIME-COURSE PROSTATE CANCER PROGRESSION IN THE DORSOLATERAL AND VENTRAL LOBES

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Running Title: A new model for induced prostate cancer

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ABSTRACT

Background: Characterization of novel rodent models for prostate cancer studies requires evaluation of either spontaneous and carcinogen-induced tumors as well as tumor incidence in different prostatic lobes. We propose a new short-term rodent model of chemically-induced prostate carcinogenesis in which prostate cancer progression occurs differentially in the dorsolateral and ventral lobes. **Methods**: Adult gerbils were treated with MNU alone or associated with testosterone for 3 or 6 months of treatment. Tumor incidence, latency, localization and immunohistochemistry (AR, PCNA, smooth muscle α-actin, p63, MGMT and E-cadherin) were studied in both lobes. **Results**: Comparisons between both lobes revealed that lesions developed first in the DL while the VL presented longer tumor latency. However, after 6 months, there was a dramatic increase in tumor multiplicity in the VL, mainly in MNU-treated groups. Lesions clearly progressed from a premalignant to a malignant phenotype over time and tumor latency was decreased by MNU+testosterone administration. Three-dimensional reconstruction of the prostatic complex showed that the DL developed tumors exclusively in the periurethral area and showed intense AR, PCNA and MGMT immunostaining. Moreover, VL lesions emerged throughout the entire lobe. MNU-induced lesions presented markers indicative of an aggressive phenotype: lack of basal cells, rupture of the smooth muscle cell layer, loss of E-cadherin and high MGMT staining. **Conclusions**: There are distinct pathways involved in tumor progression in gerbil prostate lobes. This animal provides a good model for prostate cancer since it allows the investigation of advanced steps of carcinogenesis with shorter latency periods in both lobes.

KEYWORDS: N-methyl N-nitrosurea; Testosterone; Gerbil; Prostate Cancer; Alkylating agents

INTRODUCTION

Prostate cancer is a disease of increasing significance worldwide since it is one of the most common cancers and is among the leading causes of cancer death [1]. In addition, benign prostatic hyperplasia is the most common benign neoplasm, occurring in approximately 50% of all men by the age of 60 [2].

It is difficult to investigate stages in the development of human prostate cancer, but some animal models provide opportunities in this regard [3]. Observation of the simultaneous occurrence of premalignant and malignant lesions is considered an important factor in animal models for the study of prostate carcinogenesis, since this event is indicative of progression from premalignancy to invasive cancer [4,5]. Histopathological analysis of rodent models support that this microinvasive stage, similar to high-grade prostate intraepithelial neoplasia (PIN) in humans, becomes more frequent with aging [6,7]. Thus an optimal model would develop PIN, progress to adenocarcinoma, and then metastasize to distant organs and become refractory to androgen ablation. While no single model encompasses the entire spectrum of events observed in human pathology, our understanding of the pathways involved in the etiology of this disease is based on the compilation of findings reported in different animal models [8].

Studies have shown that there are considerable differences in the ductal arrangement, histology and response to hormones between different prostatic lobes in rodent models [9-13]. Deklerk and Coffey [9] and Sugimura and colleagues [10] reported that epithelial and stromal cells from different regions of the prostate lobes respond differently to androgen deprivation and stimulation, suggesting functional heterogeneity within each lobe. Thus, the characterization of models for prostate carcinogenesis must involve the investigation of tumors arising from different regions of the gland.

Rodent models treated with chemical carcinogens to induce prostate tumors have been widely used [14]. In this regard, advanced steps of this disease may be best studied in a short period of time in these animals. In a set of several chemical carcinogens, N-methyl N-nitrosurea (MNU) has been widely used for the induction of prostate and breast tumors [15-20]. MNU is a directly acting methylating agent that reacts with cellular macromolecules, including proteins and DNA [21,22]. Its administration leads to the formation of large

amounts of O^6-methylguanine, a DNA adduct with recombinant and cytotoxic properties [23], which relates to the increased risk of cancer development [24]. However, these adducts are removed by DNA methyltransferases present in animal cells, such as O^6-methylguanine-DNA methyltranferase (MGMT) [23]. The depletion of methyltransferases results in the persistence of methylated DNA sites that are potentially mutagenic and which can trigger cancer in the target organ [22].

Short-term treatment of rats with MNU produces a low incidence (5-15%) of prostate cancer, provided that prostatic cell proliferation is enhanced during carcinogen exposure. Higher carcinoma incidence can only be produced by additional chronic treatment with testosterone [3]. After the delivery of a single tumor-initiating dose of MNU followed by subsequent long-term, low-dose testosterone treatment, there is a marked increase in prostate cancer yields, demonstrating the strong tumor-promoting activity of androgens [25]. This model has several advantages, including the development of a broad spectrum of histopathologic lesions corresponding to progression from hyperplasia to dysplasia and adenocarcinoma [17].

Our research group has employed the gerbil (*Meriones unguiculatus*) in several studies involving hormonal treatments [26,27], the development of spontaneous neoplasms associated with aging [28-31] and chemically induced tumors [32]. Satisfactory results in terms of the different experimental protocols encouraged a more detailed investigation of the onset, behavior and progress of prostate carcinogenesis in this animal.

Thus, the aim of the present study was to determine the incidence, multiplicity and tumor latency of spontaneous and chemically induced tumors in the dorsolateral and ventral lobes of the gerbil prostate. Therefore, we propose a new short-term rodent model of chemically-induced prostate carcinogenesis in which prostate cancer progression occurs differentially in the dorsolateral and ventral lobes. Further characterization of some histological markers was performed to determine the severity of the tumors and to propose this as an alternative experimental model for prostate study.

MATERIALS AND METHODS

Animals, experimental design and carcinogenesis induction

Thirty adult male gerbils (90 days) were maintained in accordance with institutional guidelines for animal treatment. The experiment was approved by the Ethics Committee of Experimental Animals of Sao Paulo State University (protocol number: 003/2009). Animals were housed in plastic cages under conventional conditions (25ºC, 40-70% relative humidity, 12 light/12 dark) in pathogen-free conditions, with water and balanced chow supplied *ad libitum*.

Induction of carcinogenesis by N-methyl-N-nitrosurea

Adult gerbils (90 days) were randomly divided into three groups: I, comprised of intact animals, MNU (N-methyl-N-nitrosurea; MNU only) and MNU+T (MNU+testosterone). Treated animals received a single intraperitoneal injection of N-methyl-N-nitrosurea (MNU; CAS 684-93-5 Sigma, St. Louis, MO; 50 mg/kg) on the first day of the experiment. The carcinogen was stored at -20ºC in the dark and the working solution was freshly prepared and dissolved in physiological saline immediately before use. Additionally, the MNU+T group received weekly doses of testosterone cypionate (2 mg/kg). All animals were killed by CO_2 inhalation after 3 and 6 months from the beginning of the experiment. Each animal was subjected to a complete autopsy and the entire prostatic complex (prostate lobes and seminal vesicles) was removed and the dorsolateral (DL) and ventral lobes (VL) were submitted to histopathological classification.

Procedures

The dorsolateral and ventral lobes of the gerbil prostate were fixed for 24 h in 4% paraformaldehyde in phosphate buffered saline, washed, dehydrated, cleared in xylene and embedded in paraffin (Histosec™, Merck, Darmstadt, Germany). Serial step sections (5 μm)

were submitted to cytochemical staining with hematoxylin and eosin for general tissue analysis before the determination of lesion multiplicity and immunohistochemical analyses.

Histopathological classification and determination of lesion multiplicity

The histopathological classification of prostate neoplasms present in the gerbil was accomplished according to previously described criteria [5]. The entire ventral and dorsolateral prostate from each animal was examined in order to quantify the number of prostatic lesions per analyzed field. This quantification was performed for the two periods of treatment to determine the number of premalignant lesions, characterized as prostatic intraepithelial neoplasia (PIN) and malignant lesions. The multiplicity was also determined for inflammatory disorders such as intraluminal and periductal inflammatory cells. All the results were submitted to statistical tests.

Three-dimensional reconstruction of prostatic complex

Three-dimensional reconstruction of gerbil prostatic complex was made to determine the spatial localization of prostate neoplasms as well as delimit the gerbil prostatic lobes. In order to do this, prostatic complex of 6 months MNU-treated animals were completely removed and processed for light microscopy. Whole prostates were serially sectioned at 5 μm and from all sections obtained 230 equidistant sections were chosen and stained with hematoxylin-eosin. The sections were analyzed with a Leica M125 stereo microscope coupled to an AxioCam HRC 10-33 photographic camera (Zeiss-Jenaval, Jena, Germany) and the images were digitalized using the software AxioVison Release 4.7.2 (Zeiss) for Windows. The software *Reconstruct* 1.0.9.9 aligned the urethra, bladder, ducts and prostatic alveoli of histological sections. After alignment, the images were processed to obtain an interface link between each section to create a three-dimensional model.

Immunohistochemistry

The antibodies used in the present study were: anti-PCNA (proliferating cell nuclear antigen) (mouse monoclonal, clone PC-10, dilution 1:50, Santa Cruz Biotechnology, CA), anti-androgen receptor (AR) (rabbit polyclonal, clone N-20, dilution 1:100, Santa Cruz Biotechnology, CA), smooth muscle α-actin (mouse monoclonal clone IA4; Santa Cruz Biotechnology, CA), p63 (mouse monoclonal clone 4A4; Santa Cruz Biotechnology, CA), O6-methylguanine-DNA methyltransferase (MGMT) (mouse monoclonal, clone E-1, dilution 1:50, Santa Cruz Biotechnology, CA) and E-cadherin (mouse monoclonal, clone 36B5, dilution 1:50, Novocastra). For the analysis, paraffin sections were deparaffinized, rehydrated through graded alcohols and antigen retrieval was performed in 10 mM citrate buffer pH 6.0, at 97ºC for 20 min. The blockade of endogenous peroxidases was obtained by covering the slides with H_2O_2 (3% in methanol) for 20 min and the blockade of non-specific protein-protein interactions was achieved by incubating sections with 3% bovine serum albumin (BSA, Sigma, St. Louis, MO). After pretreatment, the sections were incubated overnight at 4ºC with the antibodies diluted in 1% BSA. After that, slides were incubated with NovoLink Max Polymer detection system (Leica) or EnVision™+ Dual Link (Dako) and the positive signals were visualized as brown precipitates utilizing 3-3`-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) solution. Hematoxylin was used for counterstaining.

Determination of the proliferative index and percentage of AR-positive cells

Thirty-two random prostatic areas of each group immunostained for PCNA were analyzed after 6 months of treatment. Additionally, twenty random fields of each group after 6 months of treatment were immunostained for AR. In each field, the percentage of positive epithelial cells was determined relative to total cells and the results were submitted to statistical tests.

Statistical analysis

The results were checked for differences between groups, different lobes and treatment periods using the Mann-Whitney and Kruskal-Wallis tests. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed with Prism 5.0 software (GraphPad).

RESULTS

Comparisons between both lobes revealed that spontaneous premalignant lesions, represented by PIN, developed first in the DL (50% of specimens) and increased in number as a function of time (60% of specimens) (Fig. 1). Moreover, the VL took longer to develop the first premalignant lesions (Fig. 1A). Between 3 and 6 months of experiment, there was a dramatic increase in the number of spontaneous premalignant lesions in the VL that reached values similar to those found in the DL (Fig. 1A). After 6 months, 100% of the specimens developed some spontaneous premalignant lesions in the VL. Control animals did not develop malignant lesions in any of the prostatic lobes during the evaluated period (Fig. 1B). Additionally, the weight of specimens, the relative weights of the prostatic complex and prostatic lobes as well as serum levels of testosterone and estradiol did not vary significantly after treatment in the periods in question (data not shown). The animals remained healthy during the experiment and a low incidence of death was recorded after carcinogen inoculation. Furthermore, the animals maintained their normal habits of feeding and defecation, as well as low levels of stress and aggressiveness after long periods of treatment and maintenance in the animal facility.

Initiation by MNU decreased the latency of prostate neoplasms and increased the number of premalignant (Fig. 1A) and malignant lesions (Fig. 1B). MNU administration alone was able to induce numerous premalignant lesions after 3 months of treatment; however, only its association with testosterone allowed the early development of malignant lesions in both prostate lobes (Fig. 1B). As observed in the intact group, in the MNU-initiated groups, the DL developed the highest number of lesions in the first treatment period. The data also show a correlation between treatment time and increased multiplicity of lesions which progressed from a premalignant to a malignant phenotype (Fig. 1).

After 6 months, in the MNU-treated groups, premalignant (Fig. 1A) and malignant lesions increased in both lobes (Fig. 1B), reaching greater numbers in the VL. Thus, the VL appears to have a tumor latency period longer than the DL. However, an increased number of tumors developed in this lobe over time, especially under the action of MNU. Special attention should be given to the fact that the development of premalignant and microinvasive lesions of the VL and DL was stimulated by a single dose of carcinogen, even in the absence of

promotional agents such as testosterone, which reinforces the importance of this new model for prostate cancer research.

Lesions induced by MNU were more numerous and larger; however these lesions were located in the same regions occupied by spontaneous lesions (Fig. 2). The VL lesions were distributed along its entire length and commonly next to the area of lobe insertion in the prostatic urethra (Fig. 2A,C,D). In contrast, the DL lesions developed exclusively in the periurethral region in both sides of the lobe, as revealed by the three-dimensional reconstruction (Fig. 2B,C,E). No histopathological changes were seen in other regions of the prostatic complex such as the seminal vesicles, dorsal lobe or coagulating glands.

The evaluation of inflammatory disorders showed that they were mostly found in association with proliferative lesions and were frequently found in the MNU-treated groups, which had a greater number of neoplasms. A positive correlation between time and emergence of lesions was also noted (Fig. 3). Control animals also showed inflammatory infiltrates but were lower in number and extent, and appeared markedly after 6 months of treatment. In the VL the number of foci of intraluminal inflammatory cells did not vary statistically between treatments over time, but underwent an apparent decrease in the treated groups after 6 months (Fig. 3A). In the DL, this inflammatory disorder was significantly higher in the MNU group after 3 months (Fig. 3A). After 6 months, this intraluminal infiltrate increased in the MNU+T group and was lower in the other groups. After 6 months, there was a considerable increase in periductal inflammatory cells which prevailed as the most common inflammatory disorder (Fig. 3B). Similarly to intraluminal cells, the number of periductal inflammatory cells in the VL did not vary significantly between treatments over time, but underwent an increase in initiated animals after 6 months. In the DL, the number of periductal inflammatory cells was significantly higher in MNU and MNU+T groups, particularly after 6 months (Fig. 3B).

Since the period of 6 months was determined as satisfactory for the induction of a significant number of neoplasias, all of the quantitative analysis (proliferative activity and staining for nuclear androgen receptor) as well as the analysis of immunohistochemical markers (p63, smooth muscle α-actin, E-cadherin and MGMT) were verified at this time point. The proliferative activity of VL secretory cells did not show significant variations between groups, while the DL reached the highest proliferative index after exposure to MNU+T (Fig. 4G). Histopathological lesions in the VL showed fewer proliferative cells (Fig. 4A,B,C,G) than

those seen in the DL which presented large amount of PCNA-positive nuclei (Fig. 4D,E,F,G). Regardless of treatment, the DL showed the highest percentage of AR-positive cells (Fig. 5G), especially in the periurethral region (Fig. 5D-F). In the VL, AR-positive cells did not occupy a particular area, showing an apparently homogeneous distribution throughout the gland (Fig. 5A-C). Chronic androgen stimulation significantly increased the number of AR-positive cells in both prostate lobes (Fig. 5G), including neoplastic foci, suggesting the involvement of this receptor in the induction of carcinogenesis in this organ.

Additionally, in our model of induced prostate carcinogenesis, some markers that reveal the degree of aggressiveness of these lesions were also investigated. Basal cells in both prostate lobes were found below secretory cells, but did not form a continuous layer as in the human prostate (Fig. 6A). Some proliferation-induced lesions retained basal cells (Fig. 6A), whereas in some tumors, the loss of cell orientation around the secretory compartment was observed and along with spreading throughout the tumor (Fig. 6B); these are indicative factors of greater aggressiveness in these neoplasms. In premalignant lesions, a loss of cell-cell adhesions was observed in some regions, represented by the loss of E-cadherin expression (Fig. 6C), which constitutes one of the first steps towards stromal invasion. This demonstrates that, in this model, some induced lesions possess the potential to progress to an invasive phenotype. Lesions induced by chronic androgen stimulation commonly progressed to a malignant phenotype, mainly in the VL. In these cases, rupture of the smooth muscle cell layer was observed along with initial invasion toward the peritumoral stroma and clusters of tumor cells spreading in the stroma around neoplastic acini (Fig. 6D,E). Neoplasias stimulated by treatment with testosterone and the carcinogen showed a more aggressive phenotype, displaying a cribriform pattern of growth in PIN which presented high staining for MGMT (Fig. 6F-H). This enzyme is expressed in normal tissues, but its expression increases in response to DNA damage in an attempt to repair mutagenic damage. Additionally, a slight difference was noted in the staining of this enzyme between different gerbil prostate lobes; there was stronger immunostaining in the DL (Fig. 6H). Thus, the lesions studied in our model require the action of MGMT to repair sites of DNA methylation resulting from MNU initiation. If these sites are not repaired, they can become mutagenic.

DISCUSSION

The main point of the present study shows that malignant lesions can be induced in the gerbil prostate, even in the absence of an exogenous promoting agent such as testosterone. We postulated that this is due to the fact that endogenous testosterone levels are elevated in these animals, i.e. around 2500 pg/mL in five month-old animals [27,33] compared to 1865 pg/ml in six month-old Lobund-Wistar rats and 1236 pg/mL in Copenhagen rats [34]. These rat strains are considered to present a higher incidence of spontaneous prostatic neoplasms, and Wistar rats have been widely used as a model of MNU-induced prostate carcinogenesis [16,35,36]. However, tumor latency is 12 months for MNU inoculation and 8 months in MNU+T treated Wistar rats [35], considerably longer than the time points used in the current proposed gerbil model. The increased induction of tumors in MNU-treated gerbils leads us to believe that this model has an endogenous promoter which favors the induction of carcinogenesis by MNU. In future studies, we intend to evaluate the molecular pathways that promote prostate carcinogenesis in this rodent.

Furthermore, chronic androgen stimulation potentiated the development of prostate tumors in the VL and DL, mainly after 6 months, constituting a satisfactory period for prostate cancer study with sufficient incidence for statistical significance. The development of prostate cancer in different lobes reinforces the importance of this model for the study of prostate cancer. In most strains, these lesions develop in the ventral prostate lobe for which there is no homologue in humans [36]. It is desirable that malignant lesions originate exclusively from the dorsolateral prostate, because that part of the rodent prostate is identified to have the closest homology to the human prostate peripheral zone, where 70% of cancers arise [5,18]. For optimal relevance to cancer in humans, cancer in an animal model should develop spontaneously and progress through benign, premalignant and terminal refractory stages [34] and be induced in intact animals so that aspects of promotion and progression can be studied [36]. Additionally, an association between PIN and microinvasive carcinomas was observed in the current protocol and in previous studies by our group regarding induced [32] and spontaneous lesions in aged animals [29-31].

The emergence of inflammatory disorders in the prostate is an event commonly associated with the development of prostatic lesions [37,38]. There is increasing evidence

pointing to an association between prostatitis, benign prostatic hyperplasia [39-41] and human prostate cancer [37,42]. In gerbil prostatic lobes, the emergence of this inflammatory reaction was commonly associated with prostatic lesions; these were manifested initially in the DL and became more frequent in the VL after 6 months, mainly in groups treated with MNU. In malignant lesions, inflammation was rare and only scattered inflammatory cells were observed within the tumor, as reported in previous studies [38,43]. Such behavior has been reported in some invasive cancers that escape immune surveillance [44]. Moreover, in this model, progression from acute inflammation to a chronic state seems to occur with tumor development, as observed in other studies [38,43,45]. The high rates of inflammation present in intact gerbils is not well-understood, although it is believed to occur as a result of urine reflux into the gland. The presence of crystals in the lumen of prostatic acini is indicative of the occurrence of reflux. Since this animal is adapted to a desert climate, gerbils exhibit long periods of urinary retention, contributing to the establishment of urogenital tract inflammation (personal communication). Although extensive studies are needed to clarify the impact of inflammation on prostate carcinogenesis in the gerbil, is believed that it effectively contributes to the early steps of carcinogenesis but is unable to induce this pathology alone.

Although prostate lesions occurred in both lobes, DL lesions manifested exclusively in the periurethral region of the gland, while neoplasms in the VL appeared throughout the length of the lobe. One possible explanation for this preferential region for tumor development in the DL, as previously seen in Wistar and Noble rats [36,38], could be greater proliferative activity and higher concentrations of AR positive cells. Administration of MNU in a proliferative scenario makes the cells better targets for mutagenicity, leading to the establishment of tumors [16]. Moreover, as VL lesions developed throughout its length, it is reasonable to assume that this occurred due to homogenous expression of the androgen receptor and proliferating cells throughout the lobe.

Furthermore, in VL and DL lesions, the expression of MGMT was observed, which is an enzyme involved in the removal of DNA adducts as methyl groups added after initiation by MNU [22,46]. MGMT removes alkyl groups at the O^6 position of guanine in a reaction that inactivates one MGMT molecule for each repaired lesion. Endogenous MGMT expression protects mammalian cell lines from spontaneous G:C to A:T transitions, and if these sites are not repaired, they can become potentially mutagenic [46,47,48]. The higher staining for

MGMT in the periurethral region of DL suggests that this region is more susceptible to methylation. We pointed out that the addition of methyl groups possibly occurs in tumor suppressor genes, favoring the establishment of neoplasias in the DL. The expression of this enzyme was apparently lower in lesions in the VL. However, the reasons for this difference in expression between lesions in different prostatic lobes require further investigation. We hypothesize that additional pathways are involved in the development of tumors in the VL such as inactivation of MGMT at proliferative sites in the VL.

The induced lesions in the present work clearly progressed from a premalignant to a malignant state, expressing markers indicative of lesions of greater severity. The loss of E-cadherin expression or the loss of its normal localization at cell-cell contacts is consistently observed at sites of epithelial-mesenchymal transition during tumor progression. E-cadherin expression levels are often inversely correlated with tumor malignancy [49-51]. Additionally, with tumor progression, malignant cells invade the stroma, segregating the smooth muscle cells [52]. Thus, the loss of E-cadherin expression in premalignant lesions and the rupture of the smooth muscle cell layer demonstrates the progression of lesions to an invasive phenotype. Carcinomas were locally invasive lesions characterized by abnormalities in glandular architecture, cytological atypia and immunohistochemical findings such as a lack of basal cells [53] and high proliferative rates [17,54,55].

The comparative analysis of the morphological and quantitative data indicated differences regarding the incidence and tumor latency period between VL and DL in adult gerbils. The particularities observed for each prostate lobe certainly reflect differences in the histophysiology of these regions, also verified in other rodent strains [10,11]. Thus, we suggest the following general model for tumorigenesis involving both prostatic lobes of the gerbil (Fig. 7). In the present study, it was evident that the DL had a greater susceptibility to developing prostatic alterations earlier in relation to the VL under either spontaneous or chemically-induced conditions (Fig. 7 – Step 1). The pathways associated with this event involve the activation of proliferative pathways, as evidenced by the large number of PCNA-positive cells, as well as increased androgen receptor signaling, as evidenced by the greater amount of AR-positive nuclei. The VL had a longer latency period to trigger carcinogenic process, which was dependent on the androgen receptor pathway (Fig. 7 – Step 2). For the onset of VL carcinogenesis, there is an apparent need for long-term stimulation, but since this occurs with

the induction of malignant neoplasms (Fig. 7 – Step 3), it is more significant when compared to the DL (Fig. 7 – Step 4), and tumors in the VL quickly acquire a more aggressive phenotype (Fig. 7 – Step 5). It is likely that other molecular pathways are affected by treatment during the latency period which then add to the increased aggressiveness of VL neoplasms. The progression of malignant lesions initially induced in the DL to a more aggressive phenotype may or may not occur after longer periods of treatment and could involve additional activation pathways (Fig. 7 – Step 6).

Together, these findings suggest the existence of a spectrum of histopathological changes consistent with human prostate cancer progression [34], since both humans and gerbils show the premalignant state of PIN which may progress to carcinoma.

CONCLUSIONS

In summary, testosterone markedly enhanced prostate carcinogenesis, even at doses that do not measurably increase circulating testosterone. Thus, testosterone is a strong tumor promoter also in the gerbil. Premalignant and malignant lesions showed high staining for AR, a marker of androgen responsiveness. The high serum testosterone concentrations of gerbils probably accounts for the high proliferation activity and also for the high incidence of prostate neoplasms observed. Morphological changes in the gerbil prostate induced by MNU and testosterone were sequential, with premalignant lesions appearing first, followed by the development of malignant lesions. Furthermore, these lesions can arise spontaneously in the VL as well as in the DL, which is embryologically homologous to the area of greatest susceptibility to human prostate cancer. This provides a good model for prostate cancer study since it allows the investigation of advanced steps of prostatic carcinogenesis with shorter latency periods. Analysis of the expression of hormone receptors and proteins involved in prostate carcinogenesis stimulated by MNU in future studies will improve our understanding of carcinogenesis in the gerbil and better characterize the current proposed model.

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FIGURE LEGENDS

Fig. 1. Multiplicity of premalignant (A) and malignant (B) prostatic lesions in gerbil ventral and dorsolateral lobes. Statistical analysis based on the Kruskal-Wallis and Mann-Whitney tests ($P \leq 0.05$). Values are represented as mean±SEM (n=5). Superscript (*) indicates statistically significant inter-group differences regarding treatment, and superscript (§) indicates significant differences between treatment periods of the same group. The number of superscript indicates P value (one - $P \leq 0.05$; two - $P \leq 0.01$; three - $P \leq 0.001$).

Fig 1.

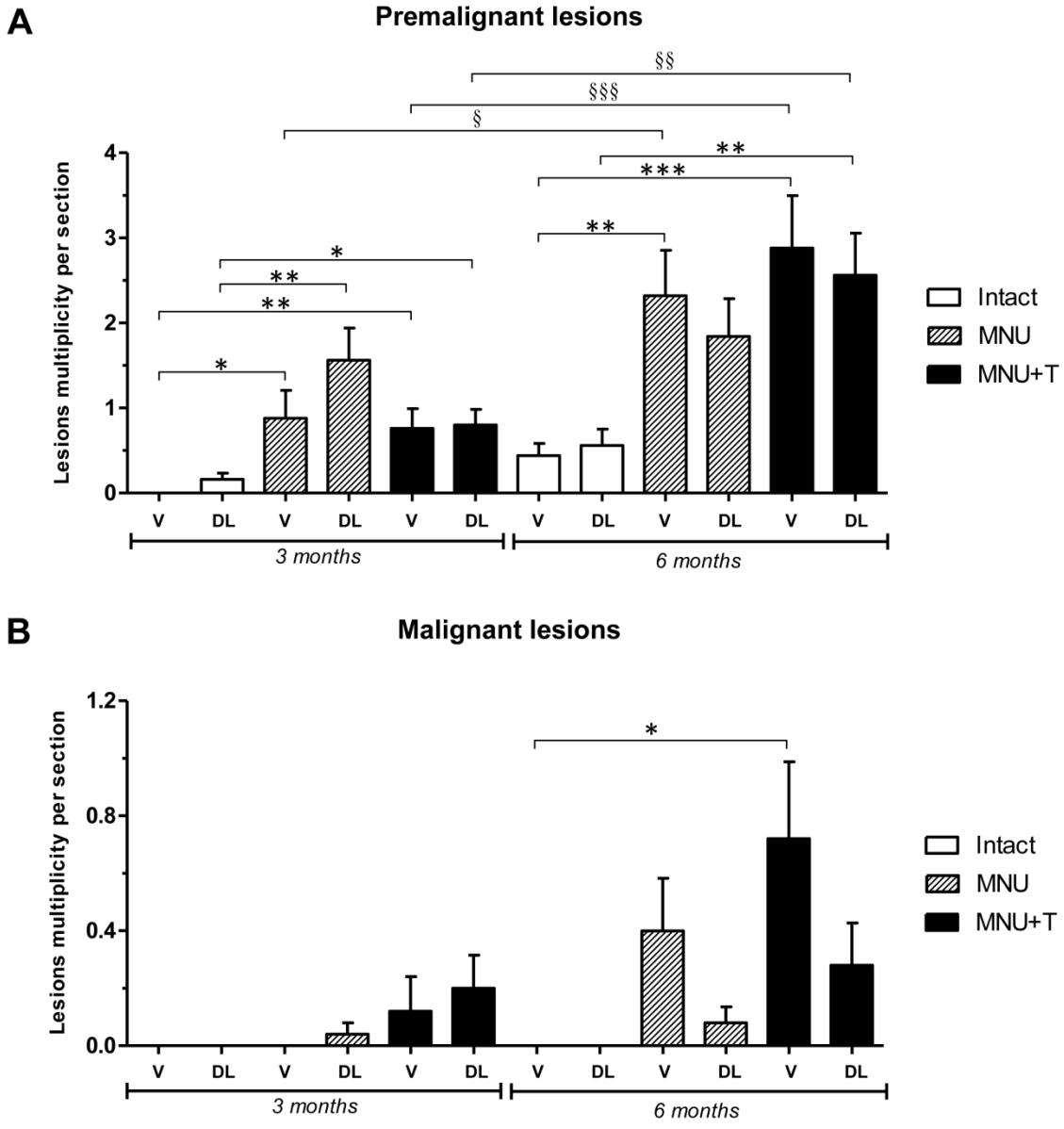

A
Premalignant lesions
Lesions multiplicity per section
0
1
2
3
4
§§
§§§
§
**
***
**
*
**
**
*
V
DL
3 months
6 months
Intact
MNU
MNU+T
B
Malignant lesions
Lesions multiplicity per section
0.0
0.4
0.8
1.2
*
3 months
6 months
Intact
MNU
MNU+T

Fig. 2. Three-dimensional reconstruction of the Mongolian gerbil's prostate complex and localization of histopathological lesions in 6 months MNU-treated animals (n=2). (A) Ventral view, (B) dorsal view and (C) lateral view of the complex. Malignant lesions of the ventral (D,F) and dorsolateral (E,G,H) prostate lobes. Note evident (thin arrows) and inconspicuous nucleoli (arrowhead), nuclei of increased size and altered form (double arrow) and mitotic figures (double arrowheads) in tumor sites of VL and DL (F, H). Microinvasion of tumor cells (thin arrows - dashed line) through the rupture of the smooth muscle layer (thick arrows) characterizing malignant lesions (G).

Fig 2.

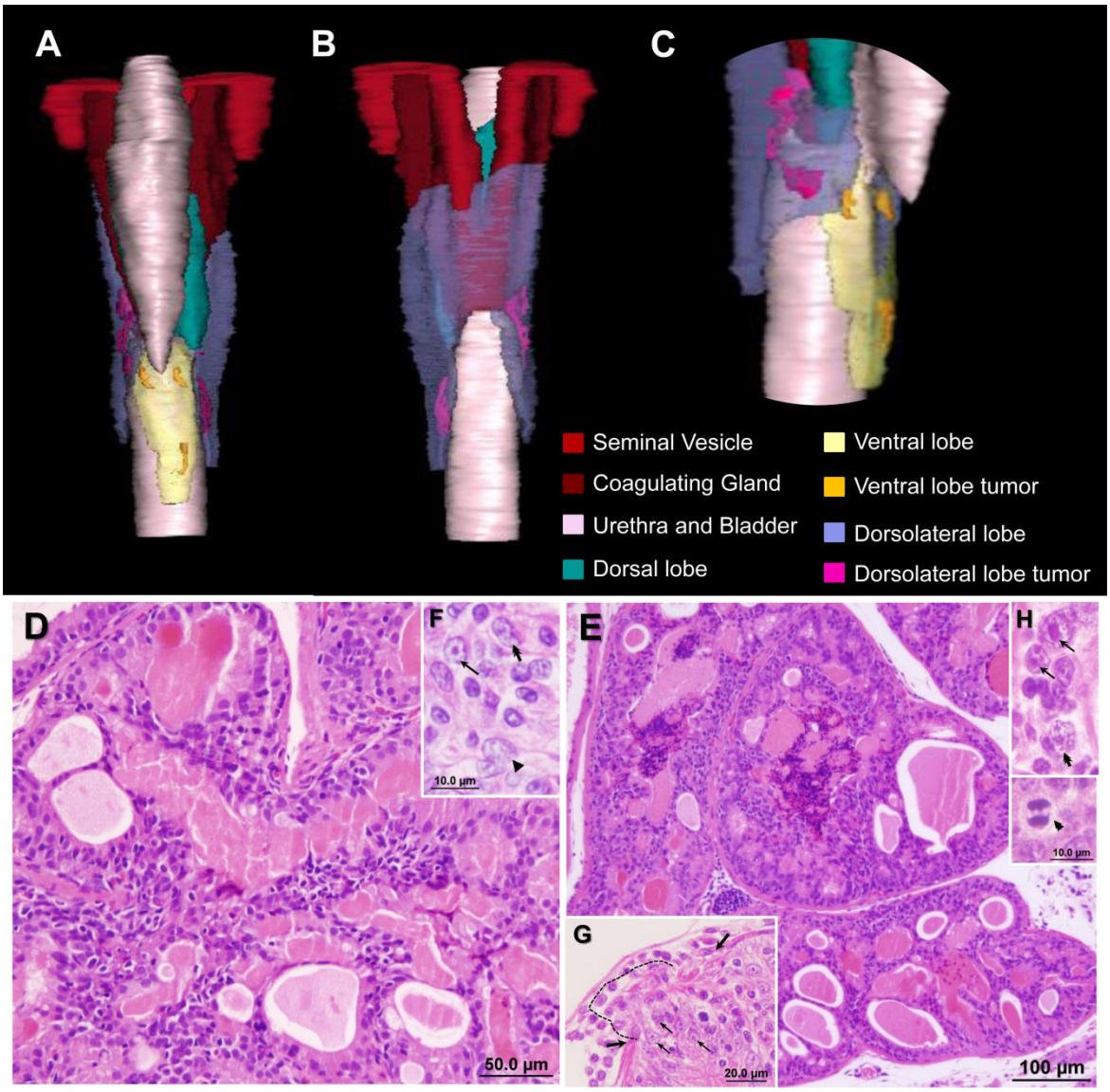

A
B
C
Seminal Vesicle
Coagulating Gland
Urethra and Bladder
Dorsal lobe
Ventral lobe
Ventral lobe tumor
Dorsolateral lobe
Dorsolateral lobe tumor
D
F
10.0 µm
50.0 µm
E
G
20.0 µm
H
10.0 µm
100 µm

Fig. 3. Multiplicity of inflammatory disorders: intraluminal (A) and periductal inflammatory cells (B) in gerbil ventral and dorsolateral prostate lobes. Statistical analysis based on the Kruskal-Wallis and Mann-Whitney tests ($P \leq 0.05$). Values are represented as mean±SEM (n=5). Superscript (*) indicates statistically significant inter-group differences regarding treatment, superscript (§) indicates significant differences between treatment periods of the same group and superscript (★) indicates significant differences between lobes of the same group. The number of superscript indicates P value (one - $P \leq 0.05$; two - $P \leq 0.01$; three - $P \leq 0.001$).

Fig 3.

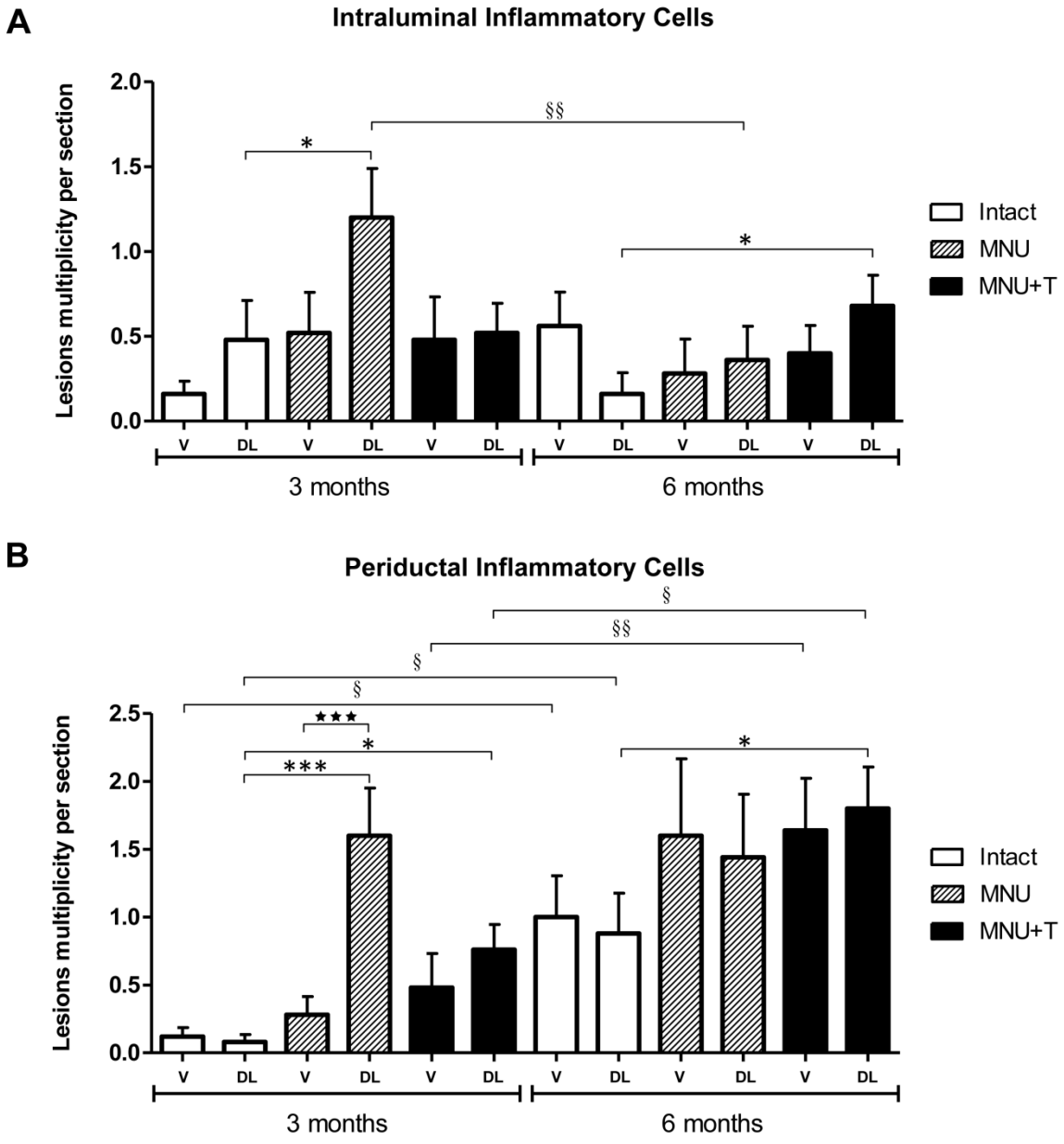

A
Intraluminal Inflammatory Cells
Lesions multiplicity per section
2.0
1.5
1.0
0.5
0.0
§§
*
*
V DL V DL V DL V DL V DL V DL
3 months
6 months
Intact
MNU
MNU+T
B
Periductal Inflammatory Cells
Lesions multiplicity per section
2.5
2.0
1.5
1.0
0.5
0.0
§
§§
§
§
★★★
*
***
*
V DL V DL V DL V DL V DL V DL
3 months
6 months
Intact
MNU
MNU+T

Fig. 4. Proliferative behavior of prostate lesions in the gerbil prostate. Proliferating cells in intact (A,E), MNU (B,D) and MNU+T groups (C,F). (F) Proliferating malignant cells in the tumor stroma (*). (G) Proliferative index of the dorsolateral and ventral prostate lobes after 6 months of treatment. Statistical analysis based on the Kruskal-Wallis and Mann-Whitney tests. Values represent mean±SEM (n=5). Superscript (*) indicates statistically significant inter-group differences regarding treatment, superscript (★) indicates significant differences between lobes of the same group. The number of superscript indicates P value (one - $P \leq 0.05$; two - $P \leq 0.01$; three - $P \leq 0.001$).

Fig 4.

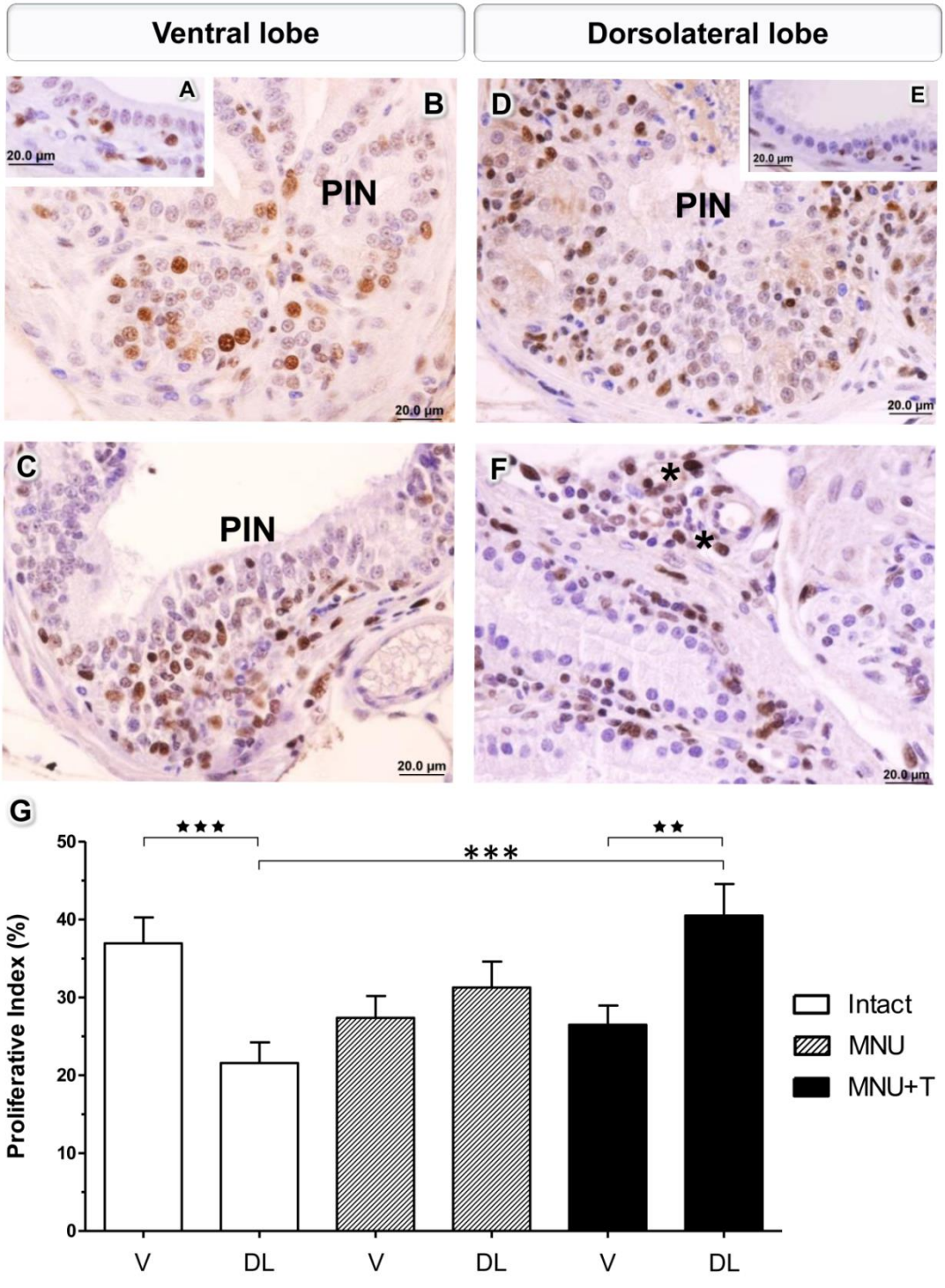

Ventral lobe
Dorsolateral lobe
A
B
C
D
E
F
G
PIN
PIN
PIN
20.0 µm
Proliferative Index (%)
0
10
20
30
40
50
V
DL
V
DL
V
DL
Intact
MNU
MNU+T

Fig. 5. AR immunohistochemistry in gerbil ventral and dorsolateral lobes. Intense AR-positive nuclei in the normal gland (A,D) and in prostate lesions (B,C,F,G). Positive staining for androgen receptor in the distal (D) and periurethral region of DL (E,F,G). (H) Determination of AR positive cells (%) in the gerbil prostate. Statistical analysis based on the Kruskal-Wallis and Mann-Whitney test ($P \leq 0.05$). Values represent mean±SEM (n=5). Superscript (*) indicates statistically significant inter-group differences regarding treatment, superscript (★) indicates significant differences between lobes of the same group. The number of superscript indicates P value (one - $P \leq 0.05$; two - $P \leq 0.01$; three - $P \leq 0.001$).

Fig 5.

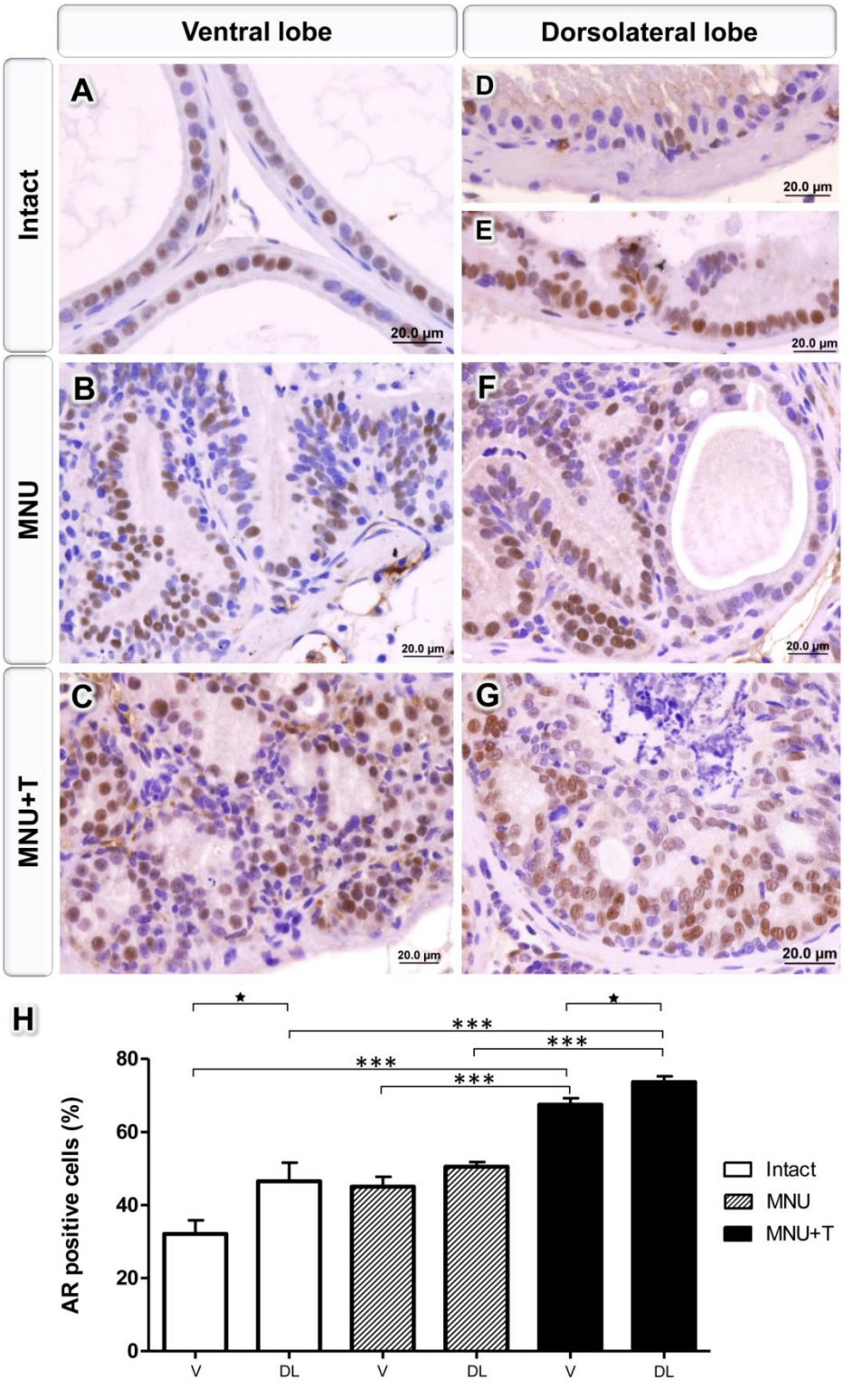

Ventral lobe
Dorsolateral lobe
Intact
MNU
MNU+T
A
B
C
D
E
F
G
20.0 µm
H
AR positive cells (%)
0
20
40
60
80
V
DL
***
Intact
MNU
MNU+T

Fig. 6. Characterization of chemically-induced lesions in the gerbil prostate. Some premalignant lesions retained basal cells which did not form a continuous layer (A), while some tumors lost basal cell orientation which spread through the tumor (arrows) (B). (C) E-cadherin staining in MNU-induced premalignant lesion in the DL (thin arrows). Some cells lost E-cadherin expression (thick arrows). (D) Clusters of tumor cells that lost the surrounding layer of smooth muscle cells (*). (E) Rupture of the smooth muscle cell layer and initial stromal invasion (arrows). PIN lesions in the VL showed weak staining for MGMT (F) while stronger immunostaining was noted in the DL (H). (G) The normal acinus (*) was negative for MGMT. MNU-induced lesions in the DL (A-C), MNU+T-induced lesion in the VL (D-G) and in the DL (H).

Fig 6.

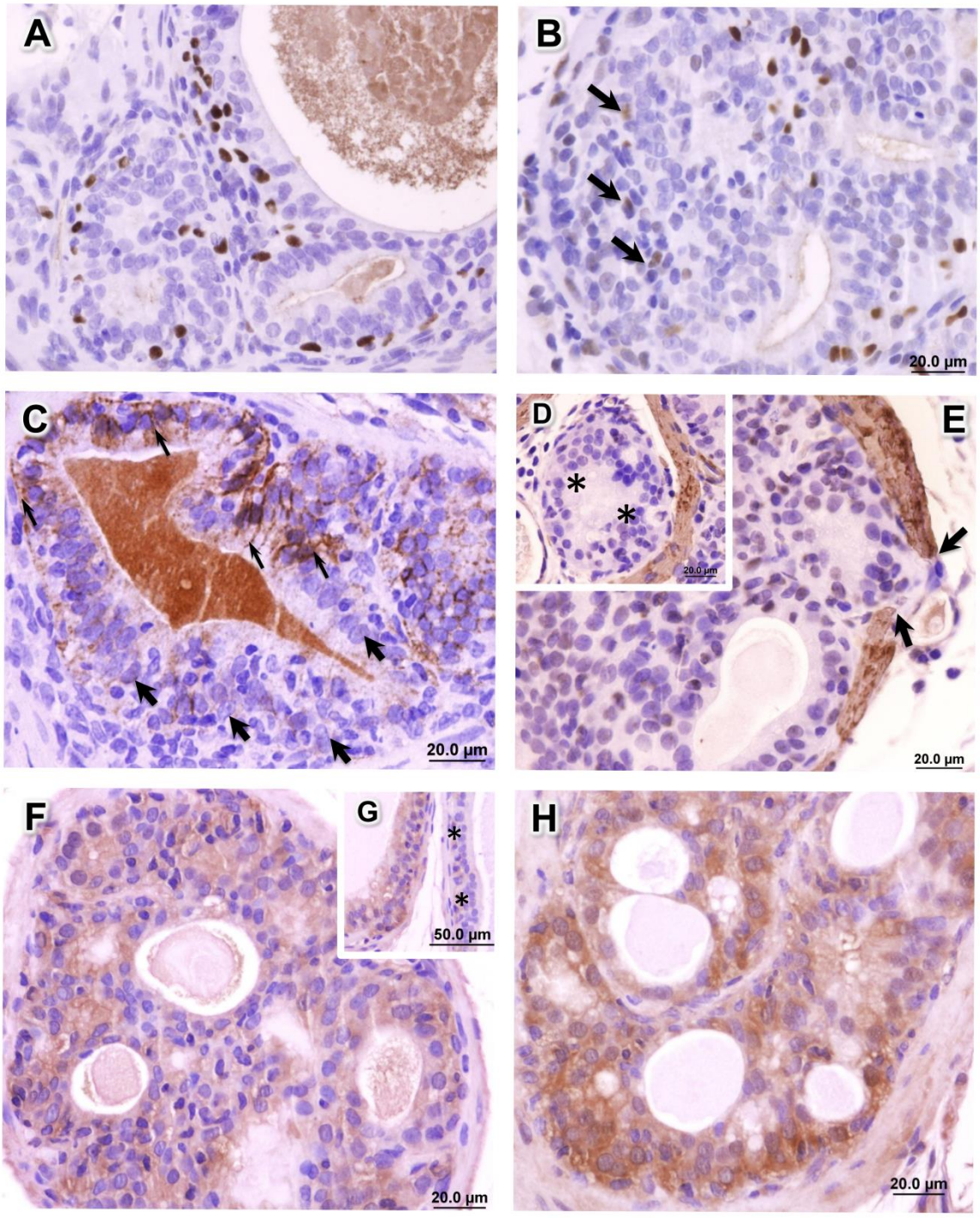
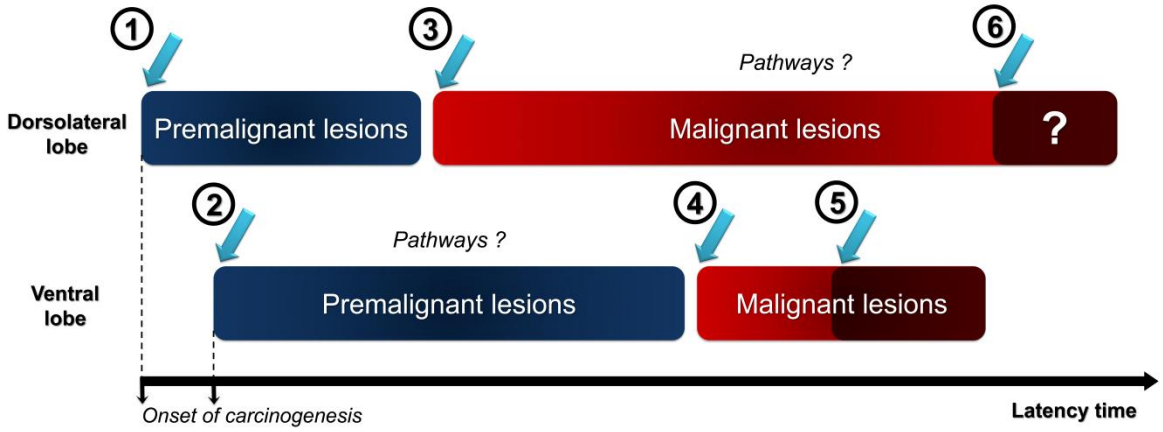


Fig. 7. General model for tumor progression involving both prostatic lobes in the gerbil. Onset of carcinogenesis in the dorsolateral ① and ventral prostate lobes ②. Malignant transformation of neoplasms in the dorsolateral ③ and ventral prostate lobes ④. The acquisition of a more aggressive phenotype quickly occurs in ventral prostate tumors ⑤ and may or may not occur in the dorsolateral prostate ⑥. During the latency period, alternative molecular pathways are possibly affected by the treatment and then contribute to distinct tumor progression processes in both prostate lobes.

Fig 7.


1
3
6
Pathways ?
Dorsolateral lobe
Premalignant lesions
Malignant lesions
?
2
4
5
Pathways ?
Ventral lobe
Premalignant lesions
Malignant lesions
Onset of carcinogenesis
Latency time

ARTIGO II

DUAL ACTION OF HIGH ESTRADIOL DOSES ON MNU-INDUCED PROSTATE NEOPLASMS IN GERBILS: THERAPEUTIC EFFECT AND EMERGENCE OF UNSTABLE EPITHELIAL MICROENVIRONMENT

Submetido para a revista

"ENDOCRINE-RELATED CANCER"

DUAL ACTION OF HIGH ESTRADIOL DOSES ON MNU-INDUCED PROSTATE NEOPLASMS IN GERBILS: THERAPEUTIC EFFECT AND EMERGENCE OF UNSTABLE EPITHELIAL MICROENVIRONMENT

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Short title: Estrogen action on induced prostate neoplasms

Keywords: Prostate cancer, Mongolian gerbil, Estradiol, N-methyl-N-nitrosurea, 5-methylcytidine, AR, p63.

Word count: 4981

Abstract

Estrogens are critical players in prostate growth and disease. Estrogen therapy was the standard treatment for advanced prostate cancer for many decades, but has currently been replaced by alternative anti-androgenic therapies. Besides many studies regarding its action on prostate biology at different stages of life, results from the association between carcinogen and estrogen are scarce and inconclusive about its beneficial role on induced-carcinogenesis. Thus, the aim of this study was to determine if estradiol exerts a protective and/or stimulatory role against N-methyl-N-nitrosurea-induced prostate neoplasms. For this purpose we adopted a rodent model which has been used for induced-prostate carcinogenesis studies, the Mongolian gerbil. Were investigated the occurrence of neoplasms, karyometric patterns, androgen receptor, basal cells and global methylation status in ventral and dorsolateral prostate. Histopathological analysis showed that estrogen was able to slow tumor growth in both lobes after prolonged treatment. However, only in dorsolateral prostate occurred a true neoplastic regression. Besides the therapeutic effects against neoplastic progression, estrogen treatment created an epithelium that exhibited distinctive features from a normal prostate as: increased androgen-insensitive basal cells, high androgen receptor positivity and changes in DNA methylation pattern. Together, these events contributed to create an unstable epithelial milieu which could trigger to lesion recurrence in subsequent periods.

Introduction

Prostate gland is a hormone-responsive organ that requires androgens to maintain its tissue homeostasis and normal growth in adulthood (Ellem & Risbridger 2009). Besides its physiological functions, androgens are known as regulators of malignant growth and are also the major target for prostate cancer prevention and treatment (Hartman *et al.* 2012). Nevertheless androgens individually are not sufficient to driver carcinogenesis and increasing evidence implicates a pivotal role for estrogens (Ellem & Risbridger 2009).

Estrogens are critical players in prostate growth regulation at all stages of life, however, its mechanisms of action during gland homeostasis and disease are still beginning to be elucidated (Ellem & Risbridger 2009). Estradiol is synthesized in low levels by the testis and locally in the prostate from androgens via the aromatase enzyme. Is generally accepted that indirect estrogen effect consists mainly in the interference in testicular androgen production by repression of hypothalamic-pituitary-gonadal axis (Scarano *et al.* 2008, Asgari & Morakabati 2011). The direct actions of estradiol on the adult prostate may be elicited by external hormones or by local aromatization of testosterone producing estradiol (Härkönen & Mäkelä 2004). These effects are mediated through the estrogen receptors -alpha (ERα) and -beta (ERβ) (Kuiper *et al.* 1996, Couse & Korach 1999).

Huggins and Hodges (1941) published a classical article which established for the first time the relationship between testosterone and prostate cancer. Additionally, they described the beneficial effects of endocrine manipulation on locally advanced and metastatic adenocarcinoma and consequently established an effective method to treat prostate cancer. From the late 1950's through the early 1980's estrogen therapy was the standard treatment for advanced prostate cancer (Cox & Crawford 1995). After the discovery of the side effects of estrogenic treatment (i.e. cardiovascular toxicity) anti-androgenic alternative therapies started to be designed, but the ability of estrogens to treat or prevent prostate cancer is under continued investigation (Prins & Korach 2008).

Despite the obstacles of estrogen therapy, the study of the action of this steroid on prostate biology at different stages of life has gained the attention of scientific community recently. Low doses of estrogens given prenatally have been shown to enhance prostatic

growth in mice (vom Saal *et al.* 1997). In contrast, high levels of estrogens given during the perinatal period elicit inhibitory effects in prostate growth, decrease responsiveness to androgens in adulthood (Naslund & Coffey 1986, Santii et al. 1991, vom Saal *et al.* 1997) and may lead to cancer (Santii *et al.* 1990, Prins 1997; 2001). Therefore it is understood that prostatic response to this steroid is dependent on many variables such as dose, exposure time and the presence of androgens (Bianco *et al.* 2002).

Prostate cancer develops through well-defined stages, from prostate intraepithelial neoplasia (considered as preneoplastic lesions), to localized (*in situ*), invasive, and finally metastatic cancer (Rouet *et al.* 2010). Because of the lengthy time between initiation and clearly invasive cancer, in most cases, early intervention and prevention strategies can potentially interrupt the cancer progression process (Umar *et al.* 2012). Many efforts have been employed in an attempt to identify agents that exert protective effects in early phases of cancer continuum. For this purpose many research groups have been investigating distinct animal models that develop spontaneous and/or induced prostate tumors.

Development of spontaneous (Pegorin de Campos *et al.* 2006, Campos *et al.* 2008, 2010, 2011) and induced (Gonçalves *et al.* 2010, 2013) premalignant and malignant lesions is frequent in gerbil (*Meriones unguiculatus*), a rodent proposed as a good model to study prostatic morphophysiology and disease. Recently our group characterized tumor incidence in different prostatic lobes from intact and MNU-induced gerbils. In this work we found that prostate cancer progression occurs differentially in dorsolateral and ventral lobes, and speculated that distinct pathways are responsible for these histopathological discrepancies (Gonçalves *et al.* 2013). Although the model of chemically-induced prostate carcinogenesis by MNU and androgen has been well characterized for gerbil (Gonçalves *et al.* 2010, 2013) and other rodent strains (Pollar & Luckert, 1987, McCormick *et al.* 1998, Liao *et al.* 2002, Boileau *et al.* 2003, Arunkumar *et al.* 2006), results from the association between carcinogen and estrogen are scarce and inconclusive about the beneficial role of this steroid on induced-carcinogenesis (Shirai *et al.*1987, Pollard *et al.*1989). This investigation may provide insights about pathogenic pathways or chemoprevention strategies for prostate cancer management.

Thus, the main objective of this study was to clarify the effect of estradiol on induced prostate cancer and contribute to a better understand of intralobular particularities that result in differential tumor incidence among distinct prostatic regions. We noticed the repercussion

of estrogenic therapy by morphometric methods, increased androgen-insensitive basal cells, high androgen receptor positivity and alteration in global methylation status, events that contributed to create an unstable epithelial milieu.

Materials and methods

2.1. Animals

Thirty adult male gerbils (100 days) were maintained in accordance with institutional guidelines for animal treatment and the experiment was approved by the Ethics Committee of Experimental Animals of Sao Paulo State University (protocol number: 003/2009). Animals were housed in plastic cages under conventional conditions (25ºC, 40-70% relative humidity 12 light/12 dark), with water and balanced chow supplied *ad libitum*.

2.2. Experimental design and carcinogenesis induction

Animals were randomly divided into three groups: C, comprised of intact animals without any intervention, MNU (N-methyl-N-nitrosurea; MNU only) and MNU+E (MNU+estradiol). Treated animals (MNU and MNU+E) received a single intraperitoneal injection of N-methyl-N-nitrosurea (50 mg/kg - CAS 684-93-5 Sigma Chemical Co.-USA) on the first day of the experiment. The carcinogen was stored at -20ºC in the dark and the working solution was freshly prepared and dissolved in physiological saline immediately before use. Additionally, MNU+E group received weekly subcutaneous doses of estradiol benzoate (Sigma Chemical Co.-USA) diluted in mineral oil (1mg/0.1mL/application). All animals were euthanized by CO_2 inhalation after 14 or 28 weeks from the beginning of the experiment. Immediately after, the entire prostatic complex (prostate lobes and seminal vesicles) was removed, weighted and dorsolateral (DL) and ventral lobes (VL) were submitted to histopathological classification.

2.3. Serum hormonal levels

Seven days after last estradiol administration blood samples were collected from each group in both treatment periods (14 and 28 weeks). The serum was separated by centrifuge at 3.000 rpm and testosterone and estradiol concentration was determined by automatic equipment (VITROS ECi-Johnson & Johnson Ultra-Sensitive Quimioluminescent analysis) in a renowned clinical analysis laboratory using specific reagents supplied by Johnson & Johnson (Amersham-UK). The values obtained were used to calculate the ratio between testosterone and estradiol levels.

2.4. Histopathological analysis

DL and VL of the gerbil prostate (n=5/group) were fixed for 24 h in 4% paraformaldehyde in phosphate buffered saline, washed, dehydrated, cleared in xylene and embedded in paraffin (Histosec™, Merck, Darmstadt-Germany). Serial sections (5 μm) were submitted to cytochemical staining with hematoxylin-eosin for general tissue analysis before histopathological classification and immunohistochemical analyses. Tissue sections were analyzed in an Olympus photomicroscope (Olympus, Hamburg-Germany) and the microscopic fields were digitalized using the software Image-Pro-Plus version 4.5 for Windows™ (Media Cybernetics Inc., Bethesda-USA).

Histopathological classification of prostate neoplasms present in gerbil was accomplished according to previously described criteria (Shappel *et al.* 2004). Five histological step sections from VL and DL of each animal (25 sections/group) were obtained in order to quantify the occurrence of histopathological lesions including: premalignant lesions, characterized as prostatic intraepithelial neoplasia (PIN) and malignant lesions. Data obtained from each group in both treatment periods were represented as lesion incidence in Table 1.

2.5 Karyometric analysis

Cell nuclear images were randomly selected from Haematoxylin-eosin paraffin sections (100x magnification) from each group at 28 weeks of treatment. Nuclear cross-sectional areas

(μm^2) and perimeters (μm) were determined for 200 epithelial secretory cells nuclei in order to obtain the form factor [= 4π.nuclear area/(nuclear perimeter)^2] parameter. The form factor parameter measures nuclear roundness and values <1 are associated with nuclei which are less round (Taboga *et al.* 2003).

2.6. Immunohistochemistry

Antibodies used in the present study were: anti-PCNA (proliferating cell nuclear antigen) (mouse-monoclonal, PC-10, dilution 1:50), anti-androgen receptor (AR) (rabbit-polyclonal, N-20, dilution 1:100), anti-p63 (transformation-related protein 63) (mouse-monoclonal, 4A4, dilution 1:100) anti-5-methylcytidine (mouse-monoclonal, 33D3, dilution 1:100,), all purchased from Santa Cruz Biotechnology (Santa Cruz, CA-USA). For the analysis, paraffin sections were deparaffinized, rehydrated through graded alcohols and antigen retrieval was performed in 10 mM citrate buffer pH 6.0, at 97ºC for 20-45 min. Blockade of endogenous peroxidases was obtained by covering the slides with H_2O_2 (3% in methanol) for 20 min and blockade of non-specific protein-protein interactions was achieved by incubating sections with 3% bovine serum albumin (BSA, Sigma, St. Louis-MO) or non-fat milk (5%). After pretreatment, sections were incubated overnight at 4ºC with the antibodies diluted in 1% BSA. After that, slides were incubated with NovoLink Max Polymer detection system (Leica) or EnVision™+ Dual Link (Dako Cytomation, CA-USA) and positive signals were visualized as brown precipitates utilizing 3-3`-diaminobenzidine tetrahydrochloride (DAB – Dako Cytomation, CA-USA) solution. Hematoxylin was used for counterstaining.

2.7. Immunohistochemical quantitative analysis

Random prostatic areas of each group, in both treatment periods, were immunostained for PCNA (32 microscopic fields/group - 40x magnification). At 28 weeks of treatment AR and p63 immunostaining (20 microscopic fields/group - 40x magnification) were performed in all groups. In each field from PCNA and AR analysis the total number of positive epithelial cells was determined. For p63 analysis the percentage of positive epithelial cells was determined relative to total cells.

Additionally, 5-methylcytidine immunostaining were conducted to determine global methylation status, and forty random fields per group (40x magnification) were used for this purpose. Specially for this analysis we used ImmunoRatio web application (Version 1.0c - Copyright© 2010-2011, Jorma Isola and Vilppu Tuominen, Institute of Biomedical Technology, University of Tampere, http://153.1.200.58:8080/immunoratio/), which has been validated for the assessment of nuclear markers in breast cancer (Tuominen *et al.* 2010, Niwas *et al.* 2013) and methylation status in glioblastomas (Burke *et al.* 2013). This plug-in calculated the percentage of DAB-stained nuclear area (labelling index) over total nuclear area by using a color deconvolution algorithm for separating the staining components (DAB/hematoxylin) and adaptive thresholding for nuclear area segmentation (Tuominen *et al.* 2010).

2.8. Statistical analysis

For comparison of results among the experimental groups, ANOVA with an a posteriori Dunnett's test (mean±SEM) (for Testosterone/estradiol ratio and PCNA) or ANOVA with an a posteriori Tukey's test (for prostatic weight, form factor, p63, AR, 5mC) were performed, according to the characteristics of each variable. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed with Prism 5.0 software (GraphPad).

Results

Quantitative and histopathological analysis allowed us to infer about the dual role of estrogen on MNU-induced prostate carcinogenesis. The effect of this steroid on tumor growth seems to be time-dependent, eliciting an inhibitory effect after prolonged therapy.

Estradiol administration significantly decreased testosterone/estradiol ratio after 14 weeks of treatment (Fig. 1). Serum levels of estradiol were the highest while testosterone levels reached the lowest values in this group (data not showed). In the same period in control and MNU-group the ratio between the two hormones remained similar. After 28 weeks, MNU-

group showed a significantly increase in this parameter compared to control group, while MNU+E maintained the lowest values.

The response of prostatic complex to treatments was similar in terms of prostatic weight and cellular proliferation (Fig. 2 and 3). Carcinogen administration alone did not affect prostate weight. Nevertheless the prostatic lobes weight decreased after 14 weeks of estradiol chronic administration (Fig. 2). However, the impact of estradiol to reduce the size of the gland and its proliferative activity became effective only after 28 weeks of treatment.

After 14 weeks of treatment a larger number of proliferative cells were verified in prostatic epithelium compartment of MNU and MNU+E group compared to control group (Fig. 3A). Moreover the response of VL and DL to treatments was similar in terms of prostatic weight and cellular proliferation. Even though a slightly decrease in MNU+E in relation of MNU was observed, cell proliferation in both groups remained similar. Prolonged exposure to estradiol reduced the number of PCNA positive cells to values similar to control group (Fig. 3B). MNU treatment for 28 weeks showed its effective action on cell kinetics of epithelial cells, promoting a great increase in the proliferative index.

In a general manner, treatment with estradiol reduced the incidence of premalignant and prevented malignant lesions in the gerbil prostate (Table 1). In contrast with the columnar simple normal epithelium (Fig.4A,F), premalignant lesions were represented by PIN which was characterized by cellular stratification with anomalous proliferation, loss of cellular polarity (Fig.4B,D), marked nuclear pleomorphism, evident nucleoli and heterogeneous chromatin texture and distribution (Fig.4C). This premalignant state was confined to the acinar outline and had not invaded the surrounding stroma. On the other hand, malignant lesions showed neoplastic cells invading the adjacent stroma trough the rupture of basement membrane and smooth muscle layer (Fig.4E), characterizing microinvasive lesions. Additional malignant lesions were identified (Fig.4G,H) exhibiting larger proportions and associated with inflammatory foci (Fig.4H), however there was only local invasion without evidence of metastases.

After 14 weeks of treatment the occurrence of premalignant lesions in MNU+E group decreased in VL and remained similar to MNU-group for DL (Table 1). However, for both lobes values from MNU+E group overcame the control group. After 28 weeks was observed in VL a slow growth of lesions induced by MNU after continuous exposure to estrogen and a

true neoplastic regression in DL (Table 1). In the other groups, in the same period, there was an increase of lesions mainly in VL and progression from premalignant to malignant state exclusively in MNU group.

The results suggest that after prolonged estradiol treatment there was a recovery from effects caused by MNU administration, i.e. high proliferative index and development of premalignant neoplasms. In order to better characterize prostate gland after prolonged hormone exposure, some quantitative and immunohistochemical analysis were performed (Fig. 5-8).

Karyometric data showed similarities between nuclear phenotypes of control and MNU+E groups (Fig. 5A). Form factor values of these groups indicated that the nuclei exhibited shape closer to circular (Fig. 5A-C,F,G) as opposed to the irregularly shaped atypical nuclei often found in MNU-group (Fig. 5D-E). A homogeneous pattern of chromatin distribution is present in nuclei of control and MNU+E group (Fig. 5F,G) and nucleolar corpuscles are less evident. Nuclei observed in MNU-group showed heterogeneous chromatin texture, bizarre shapes, enlarged size, large and conspicuous nucleoli (Fig. 5D,E).

Additionally, it was observed increased frequency of basal p63-positive cells in both lobes of MNU+E group (Fig. 6A,D,E). MNU administration did not alter the frequency of basal cells in any of the lobes analyzed, which remained similar to values observed in control group (Fig. 6A-C). Proliferative lesions remaining after estrogenic therapy maintained the basal cell layer intact, demonstrating non-invasive phenotype of neoplasms (Fig. 6E). On the other hand, lesions induced by single MNU administration progressed through 28 weeks acquiring invasive properties, as evidenced by the discontinuity of the basal cell layer (Fig. 6B).

In the epithelial compartment the frequency of AR positive cells was increased in groups that received the carcinogen alone or associated with estrogen (Fig. 7A). Comparisons between lobes showed that after estrogen treatment there was a tendency for reduction in number of positive AR cells in DL (Fig. 7A,F,G), while in MNU-group values were similar between both lobes (Fig. 7A,D,E). The density of AR positive cells was greater in neoplastic areas (Fig. 7D-E) than in normal epithelium (Fig. 7B-C).

Global methylation status of prostate epithelium was determined through the 5-Methylcytidine (5mC) immunolocalization (Fig. 8A-G). Differences in the intensity of immunostaining could be detected by ImmunoRatio web application. Analysis from VL

revealed a labeling index of 14% in MNU-group versus 46% of methylation in MNU+E group. Compared to control prostate MNU-treated epithelium suffered hypomethylation (Fig. 8A,D), while in MNU+E group hypermethylation was noticed (Fig. 8A,F). In DL, the level of methylation remained similar to that seen in the VL of MNU+E group (Fig. 8A,G). However, DL prostate epithelium, sensitized exclusively by MNU, presented an augment in 5mC (Fig. 8A,E).

Discussion

Since there are several evidences showing pro and anti-tumor role for estrogens in prostate, the present study was designed to investigate its possible dual role on gerbils' prostatic lobes after initiation by MNU. In our current model, prostate carcinogenesis is stimulated by a single carcinogen administration to produce neoplasms after 14 weeks of treatment (Gonçalves *et al.* 2013). In this scenario we provided a continuous high-dose estrogen exposure throughout the necessary period for establishment of neoplasms (i.e. 14 weeks) and its progression to advanced stages (i.e. 28 weeks).

Several classes of estrogen were used as the primary medical treatment for metastatic prostate cancer for many years but have been superseded by luteinizing hormone-releasing hormone agonists and antiandrogens due its adverse effects such as cardiovascular toxicity (Oh 2002). The basis of high-dose estrogen therapy for prostate cancer was its indirect anti-androgen action mediated through feedback inhibition of hypothalamic luteinizing hormone and pituitary luteinizing hormone release, resulting in decreased testicular androgen synthesis and release, i.e. chemical castration (Nelles *et al.* 2011). Besides new therapies have been used to control prostate tumor growth, the ability of estrogens to treat or prevent prostate cancer is under continued investigation (Prins & Korach, 2008).

Our results showed that since the beginning of the treatment testosterone/estradiol ratio decreased drastically in MNU+E group as a result of increased estradiol and lower testosterone levels. This change in hormone levels is probably the basis for prostatic lobes weight reduction and lower incidence of neoplasias noted. Fencl & Ville (1973) affirmed that the mechanisms involved in diminishing the growth of the prostate after estradiol

administration and castration are different, although both procedures lead to a decreased level of circulating testosterone. However, surgical castration reduces only testosterone serum levels derived from testis, while estradiol therapy appears to further reduce intratumoral androgens, resulting in tumor regression (Montgomery *et al.* 2010). Some investigators have suggested that estrogens directly inhibit growth of prostate cancer when administered *in vitro* in the absence of circulating hormones (Robertson *et al.* 1996) and in human prostate cancer xenografts in castrated mice (Montgomery *et al.* 2010).

The data presented here suggest that inhibitory effects of estrogen therapy on tumor establishment and growth were related with time of exposure. In early stages of the treatment were observed neoplasms manifesting in both prostatic lobes and a high proliferative index, but with prolonged therapy there was a reduction of proliferation and in incidence and growth of prostate lesion. The antitumor effects mediated by estrogen are not limited to interference in androgenic status of prostate gland (Ho *et al.* 2011). Estrogens are predicted to produce antiproliferative and antiangiogenic compounds trough *in vivo* metabolism. This evidence could explain the anti-prostate cancer effect of estrogens besides their ability to suppress endogenous androgens (Scherr *et al.* 2003) and reflects their relevance in patients with advanced prostate cancer which in most cases are insensitive to antiandrogenic therapies (Prins & Korach, 2008). In MNU group, which developed the highest incidence of neoplasias, testosterone/estradiol ratio remained high. Testosterone is known to stimulate mitotic activity of prostatic cells, which after carcinogenic insult by MNU leads to the emergence of neoplasms (McCormick *et al.* 1998, Gonçalves *et al.* 2010, 2013). This leads us to speculate that the initial high incidence of premalignant lesions in MNU+E group was due the residual tissue androgens which following continuous high-dose estrogen treatment was suppressed. After 28 weeks, when androgen levels fell, tumor growth reduction became more effective.

In a study using Lobund-Wistar rats inoculated with MNU and exposed to slow-release testosterone implants, Pollard *et al.* (1986) reported that treatment with estradiol at intermediate points in the projected latency period of tumor development significantly slows prostate cancer development. On the other hand, there was no therapeutic benefit by estradiol among advanced tumors (Pollard *et al.* 1986). Previous studies of our group found that estrogen leads to epithelial proliferative disorders as prostatic hyperplasia, PIN as well as dysplasia (Scarano *et al.* 2008). However, in this work animals were treated for 21 days and the

incidence of lesions was not quantified. Moreover, prostatic response to this steroid depends on additional variables such as dose, exposure time and the presence of androgens (Bianco *et al.* 2002). The rationale behind these data is that early stages in induced prostate tumorigenesis are sensitive to modulating agents. In particular, during tumor progression from high grade PIN to prostate cancer, specific changes of gene expression in epithelial and stromal tumor cells contribute to enhance the tumor cell growth, survival, migration and invasiveness (Mimeault & Batra 2006; Ho *et al.* 2011). Intervention in early points of tumoral process may be more effective in avoiding growth of cancer cells since it can prevent successive genetic and epigenetic changes required for malignancy (Umar *et al.* 2012).

In general, the effects mediated by estrogen administration were similar between prostatic lobes, but some particularities were found. Our group has reported that signaling pathways should be operating differently between prostatic lobes to enhance or inhibit tumorigenesis**.** The study showed that the tumor latency period for DL is shorter while progression to more aggressive stages is faster in VL (Gonçalves *et al.* 2013). The current work demonstrates that estradiol slows tumor growth in both lobes, indeed only in the DL there was an effective regression of lesions. Taken together these evidences reinforce the idea that in VL some molecular pathways are activated during carcinogenic processes supporting cancer cells growth and survival (Gonçalves *et al.* 2013). Nevertheless, the VL seems to be more sensitive to hormonal imbalances and carcinogenic insults and have less ability to transpose these adversities eliminating cancer cells. In contrast, in DL these pathways are not activated and/or some protective factor (i.e. increased expression of DNA repair enzymes) acts in order to control neoplastic growth.

Nuclear architecture is a key factor in cell functioning and pathogenesis. Alterations in nuclear structure are very relevant to clinical and research fields, especially in the context of early cancer detection (Zink *et al.* 2004, Misteli, 2005). We reported here the presence of atypical nuclei i.e. increase in nuclear perimeter, bizarre shapes, and conspicuous nucleoli after MNU administration. Genetic and epigenetic abnormalities implicated in cancer initiation and progression inevitably alter the nuclear landscape, resulting in shape, size and textural irregularities in the nucleus (Nandakumar *et al.* 2011). These alterations have been implicated as a diagnostic factor to discriminate between normal cells, benign prostatic hyperplasia and prostate adenocarcinoma (Taboga *et al.* 2003). Estrogen therapy caused a recovery of nuclear

phenotype similar to normal cells, indicating that metabolic state of prostatic nuclei remained similar to the normal tissue.

Intending to understand the differences in tumor growth between prostatic lobes and to propose an explanation of how estradiol exerts a protective role against prostate cancer, we analyzed the frequency of basal cells, AR status and global methylation pattern throughout 5mC immunohistochemical analysis after prolonged high-exposure to estradiol.

The results showed an augment of basal p63-positive cells after estrogen therapy in both lobes. Basal cell-like phenotypes are characterized by their androgen-independence due to the lack of AR and significant expression levels of K5, K14, p63, antiapoptotic Bcl-2 protein and telomerase (Mimeault & Batra 2006). Androgen deprivation results in a dramatic reduction of a population of prostate secretory cells that are androgen-dependent, through apoptosis (Heinlein & Chang 2004). In recent work, Campos *et al.* (2013) characterized prostatic epithelial cells remaining after hormone ablation in aged gerbil. They showed that glandular epithelium resists after castration and suggests that basal cells are responsible for its renewal. Accordingly to Campos *et al.* (2013) and others (Arcolino *et al.* 2010 Jing *et al.* 2013) we speculated that, at intermediate steps in estrogen treatment, prostatic luminal cells underwent apoptosis and after prolonged exposure, as an attempt to reconstitute the normal prostatic epithelium, basal cells replenished themselves.

In particular, this "recovered" epithelium presented some particularities, i.e, it exhibited high density of AR positive cells, similarly observed in MNU group. Increased AR positivity is not correlated with the regression of prostate cancer in androgen depleted environment. After androgen deprivation some patients invariably relapse with a more aggressive form of prostate cancer, termed castration resistant (CRPC). In most cases of CRPC AR is expressed at high levels, and these tumors resume their expression of multiple AR-regulated genes, indicating that AR transcriptional activity becomes reactivated at this stage of the disease (Yuan & Balk 2009). We hypothesized that estrogen can be beneficial in the early stages of prostate carcinogenesis. In contrast, after prolonged treatment an adjustment of the gland can occur in response to androgen deprivation conditions, and prostatic cells became sensitive to low concentrations of androgen. We also speculated that pathways involved in activation of AR remains active even after estrogen therapy. Furthermore, studies employing the tumor induction model based on MNU and testosterone association showed high rates of AR

positive cells in both VL and DL of Wistar rats (Liao *et al.* 2005) and gerbils (Gonçalves *et al.* 2013). Together, with the present data this evidence indicates that carcinogen probably acts on androgen pathway to favor its activation. Since AR pathway has decisive impact on promotion and progression of prostate cancer (Lavery & Bevan 2011) is reasonable to imagine that the continuity of estrogen treatment applied to this model could lead to the development of higher grade prostatic lesions in future steps.

DNA methylation is critical for regulation of multiple cellular events and so has been implicated at a global and local level in carcinogenesis (Sharma *et al.* 2010). For this purpose we investigated if 5mC status, a marker of DNA methylation levels (Chen *et al.* 2013), was altered among treatments and lobes analyzed and if it correlates with neoplastic growth observed for different groups. Regarding estrogen treatment, it leads to overall prostate epithelium hypermethylation in both analyzed lobes as previously described in Wistar rats (Augusto *et al.* 2011).

On the other hand, when carcinogen was administered alone, VL epithelium suffered hypomethylation while DL showed an augment in 5mC immunostaining. DNA hypomethylation is proposed to cause activation of oncogenes and genetic instability, whilst hypermethylation is associated with inappropriate gene silencing (Sharma *et al.* 2010, Jerónimo *et al.* 2011). Hypomethylation induces an open chromatin structure which facilitates gene transcription, whereas a closed structure inhibits transcription (Patel *et al.* 2013). This clearly explains the low incidence of lesions observed in the group of estrogenized animals and the increasing rates of tumors in MNU group, especially VL. Additionally, we hypothesized that hypomethylation in VL of MNU-group allowed the activation of genes involved in AR signaling. Exploration of molecular mechanisms underlying these observations may provide insight into the comprehension of prostatic regional differential sensitivity to carcinogens. Additionally, increased methylation in DL of MNU-group provided an important data about the particularities observed between the lobes. In a previous report of our group it was assumed that DL proliferative lesions requires long-term stimulation to progress to more aggressive malignant states and a reasonable explanation would be the involvement of signaling pathways operating differentially between lobes (Gonçalves *et al.* 2013). The present data suggest that genes involved in cell proliferation and survival undergo methylation in this lobe, which would ensure protection against development and tumor progression.

Under the present conditions we propose the following scheme to understand the histopathological and molecular events that take place during MNU-induced carcinogenesis followed or not by estrogenic therapy (Fig. 9). After tumor induction by MNU histopathological lesions with high proliferative index develop trough latency period and can be seen in both conditions after 14 weeks (Fig. 9 – Step 1). Throughout the course of estrogen treatment, the growth rate of the lesions is reduced and some involute and disappear, while tumor growth and progression are maintained without further treatment (Fig. 9 – Step 2). After 28 weeks, it was observed that estradiol stimulates prostatic epithelium hypermethylation similarly between the lobes. However, which possibly explains the neoplastic regression of DL is the lower AR positivity. The persistent VL lesions in this group are possibly due increasing trend of AR positivity. Thus we speculate that androgen pathway remains active in this lobe and stimulates the survival of cancer cells (Fig. 9 – Step 3). On the other hand, when no further treatment is administered following MNU there was a similar increase in AR signaling in both lobes. So the differences observed between lobes in this group should not be directly driven by AR but related to the variation of global methylation between them (Fig. 9 – Step 4). Since DL presented higher status of methylation, we assume that the genes involved in the process of cellular proliferation and survival are inactive, contrary to what occurs in the VL in which the hypomethylation allows transcription of these genes favoring tumorigenesis.

Conclusion

In summary, this study has shown that long exposure to high-doses of estradiol is able to prevent the development of prostate cancer and slows tumor growth. However, the "recovered" epithelium exhibited distinctive features from a normal prostatic epithelium as: increase in the number of basal cells, high AR positivity and changes in DNA methylation pattern. These markers indicate the possibility of the lesion recurrence in subsequent periods and represent the genesis of an unstable epithelial microenvironment. Additionally, data from this work contribute to the characterization of the model recently proposed by our group, the Mongolian gerbil, and for understanding intralobular particularities that result in differential tumor incidence in many rodent strains. Together, these findings denotes the putative dual role

of estrogen on prostate carcinogenesis and show the need for further studies in this area to clarify the complex action of this steroid in prostate physiology and disease.

Declaration of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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Figure Legends

Figure 1 Ratio betweem testosterone and estradiol serum hormonal levels from experimental groups after 14 and 28 weeks of treatment. Values represented in the column bars represent the mean. Values are represented as mean±SEM (n=5). Statistical analysis based on the one-way ANOVA followed by Dunnett's multicomparison test. Superscripts indicate statistically significant difference (* P≤0.05; ** P≤0.01).

Table 1 Occurrence of Premalignant and Malignant prostatic lesions in gerbil prostate.

Groups	*14 weeks*				*28 weeks*			
	Premalignant		Malignant		Premalignant		Malignant	
	Ventral Lobe	Dorsolateral Lobe	Ventral Lobe	Dorsolateral Lobe	Ventral Lobe	Dorsolateral Lobe	Ventral Lobe	Dorsolateral Lobe
C	(1/25) - 4%	(4/25) - 16%	(0/25) - 0	(0/25) - 0	(8/25) - 32%	(8/25) - 32%	(0/25) - 0	(0/25) - 0
MNU	(7/25) - 28%	(15/25) - 60%	(0/25) - 0	(1/25) - 4%	(15/25) - 60%	(14/25) - 56%	(5/25) - 20%	(2/25) - 8%
MNU+E	(2/25) - 8%	(15/25) - 60%	(0/25) - 0	(0/25) - 0	(3/25) - 12%	(2/25) - 8%	(0/25) - 0	(0/25) - 0

The data were expressed as absolute number of affected section whereas occurrence percentages.

Figure 1.

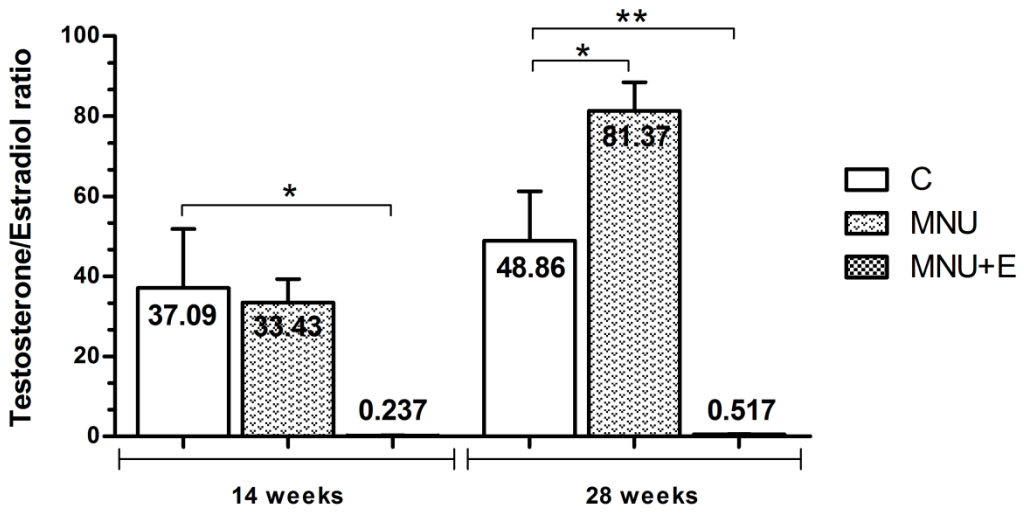


Figure 2 Prostate complex weight (g) from experimental groups after 14 and 28 weeks of treatment. Values are represented as mean±SEM (n=5). Statistical analysis based on ANOVA and Tukey-honest test. Superscripts indicate statistically significant difference (** P≤0.01; *** P≤0.01).

Figure 3 Proliferative index of the dorsolateral and ventral prostate lobes after 14 (A) and 28 (B) weeks of treatment (Number of PCNA-positive cells/field). Values are represented as mean±SEM (n=5). Statistical analysis based on the one-way ANOVA followed by Dunnett's multicomparison test. Superscripts indicate statistically significant difference (* P≤0.05; ** P≤0.01; *** P≤0.01).

Figure 2.

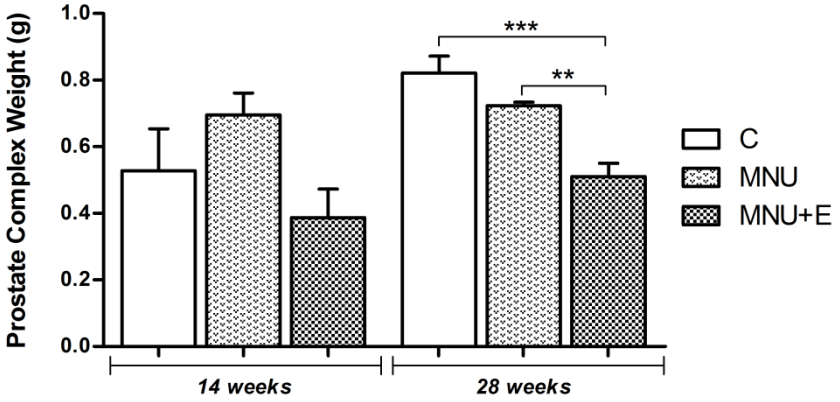


Figure 3.

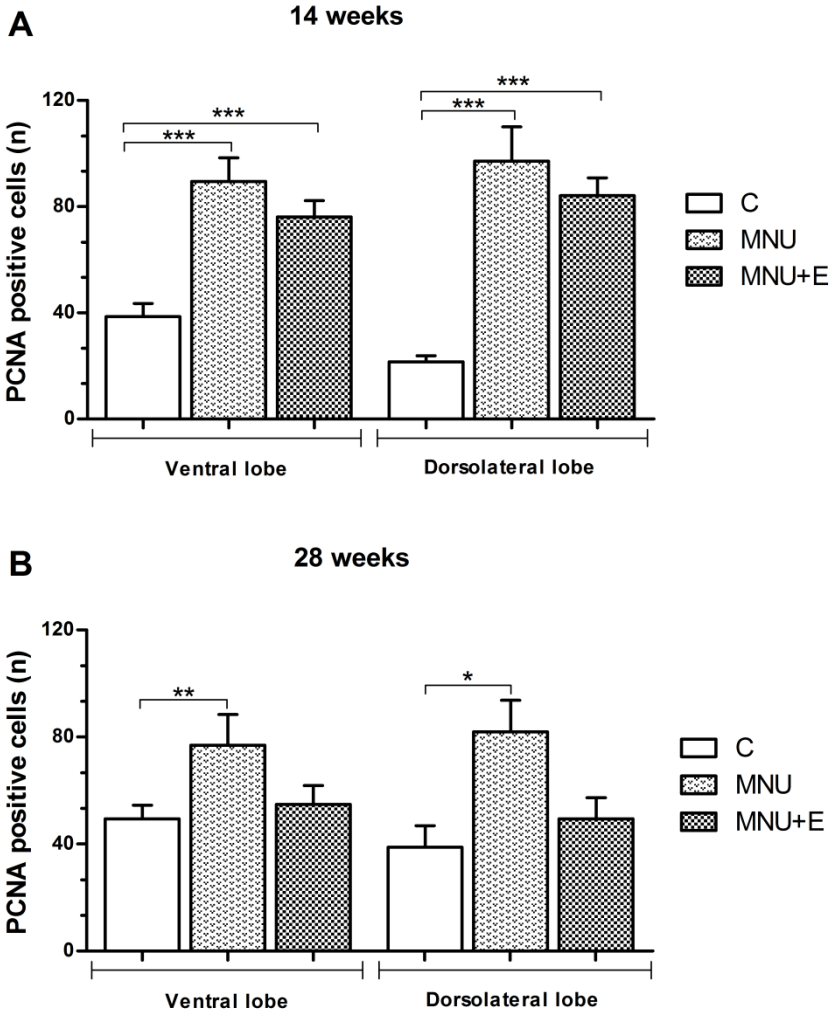


Figure 4 Characterization of chemically-induced lesions in the gerbil prostate. Normal epithelium from (A) ventral and (F) dorsolateral lobe. (B,D). Prostate intraepithelial neoplasia (PIN) was characterized by intense cellular stratification with anomalous proliferation, loss of cellular polarity, (C) intense nuclear pleomorphism, evident nucleoli (arrowheads) and heterogeneous chromatin texture and distribution. (E,G) Malignant lesions were characterized by the rupture of basement membrane and smooth muscle layer (arrows) whereby cancer cells could invade adjacent stroma. (H) Invasive lesions exhibiting larger proportions and association with inflammatory foci, however there was only local invasion without evidence of metastases. (MC) microinvasive carcinoma, (*) inflammatory foci, (Ep) epithelium, (S) stroma, (L) lumen. A-C,E,G,H – Ventral lobe; D,F – Dorsolateral lobe; A,F – Control group; B-C,E,G,H – MNU group; D – MNU+E group.

Figure 4.

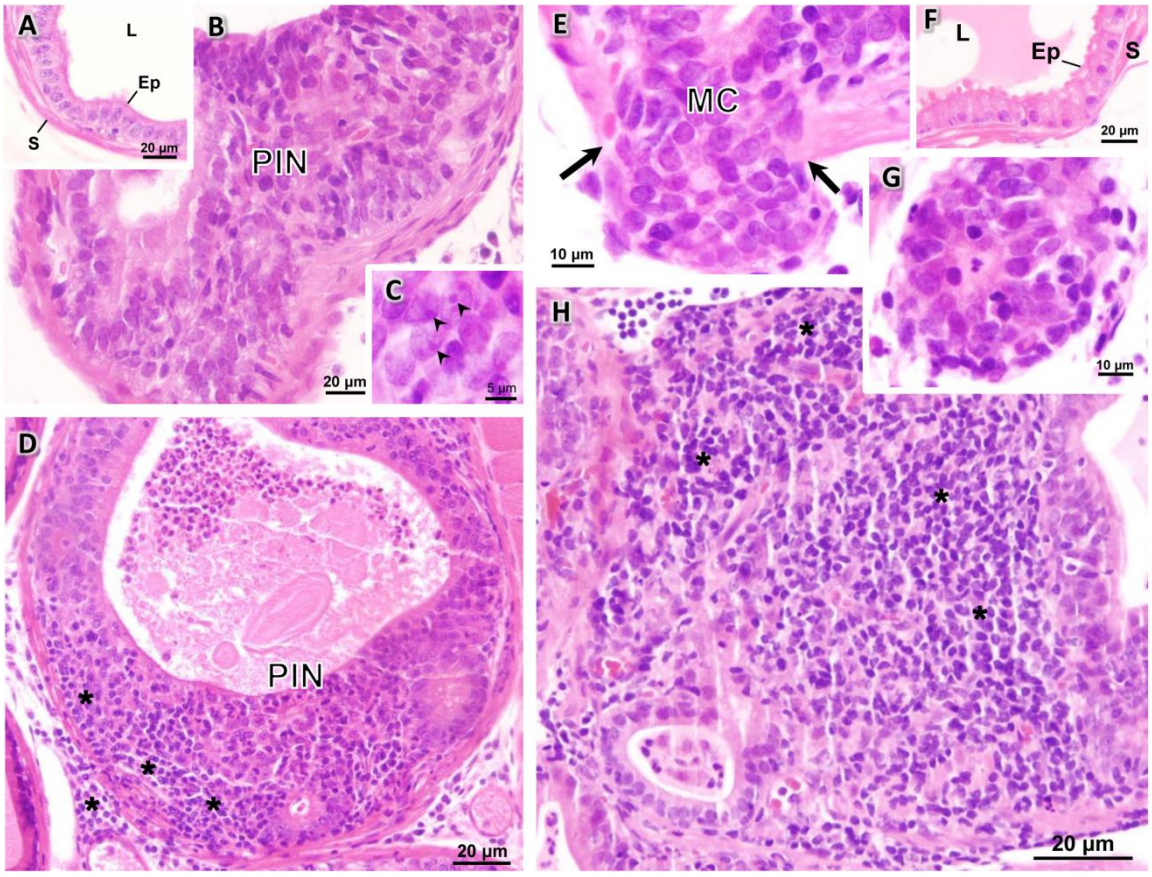

A
L
Ep
S
20 µm
B
PIN
20 µm
C
5 µm
D
PIN
20 µm
E
MC
10 µm
F
L
Ep
S
20 µm
G
10 µm
H
20 µm

Figure 5 Karyometric data. (A) The form factor parameter [=4π.nuclear area / (nuclear perimeter)^2] measures nuclear roundness of gerbils' ventral and dorsolateral prostate after 28 weeks of treatment and values <1 are associated with nuclei which are less round. Statistical analysis based on ANOVA and Tukey-honest test. Values are represented as mean±SEM (n=5). Superscript (***) indicates P value ≤0.001. (B,C) Normal epithelium from control group; (D,E) Atipical neoplastic nuclei from MNU group showed evident nucleoli (arrows) and bizarre shapes; (F,G) Epithelium similar to normal in MNNU+E group. Scale bars = 5μm.

Figure 5.

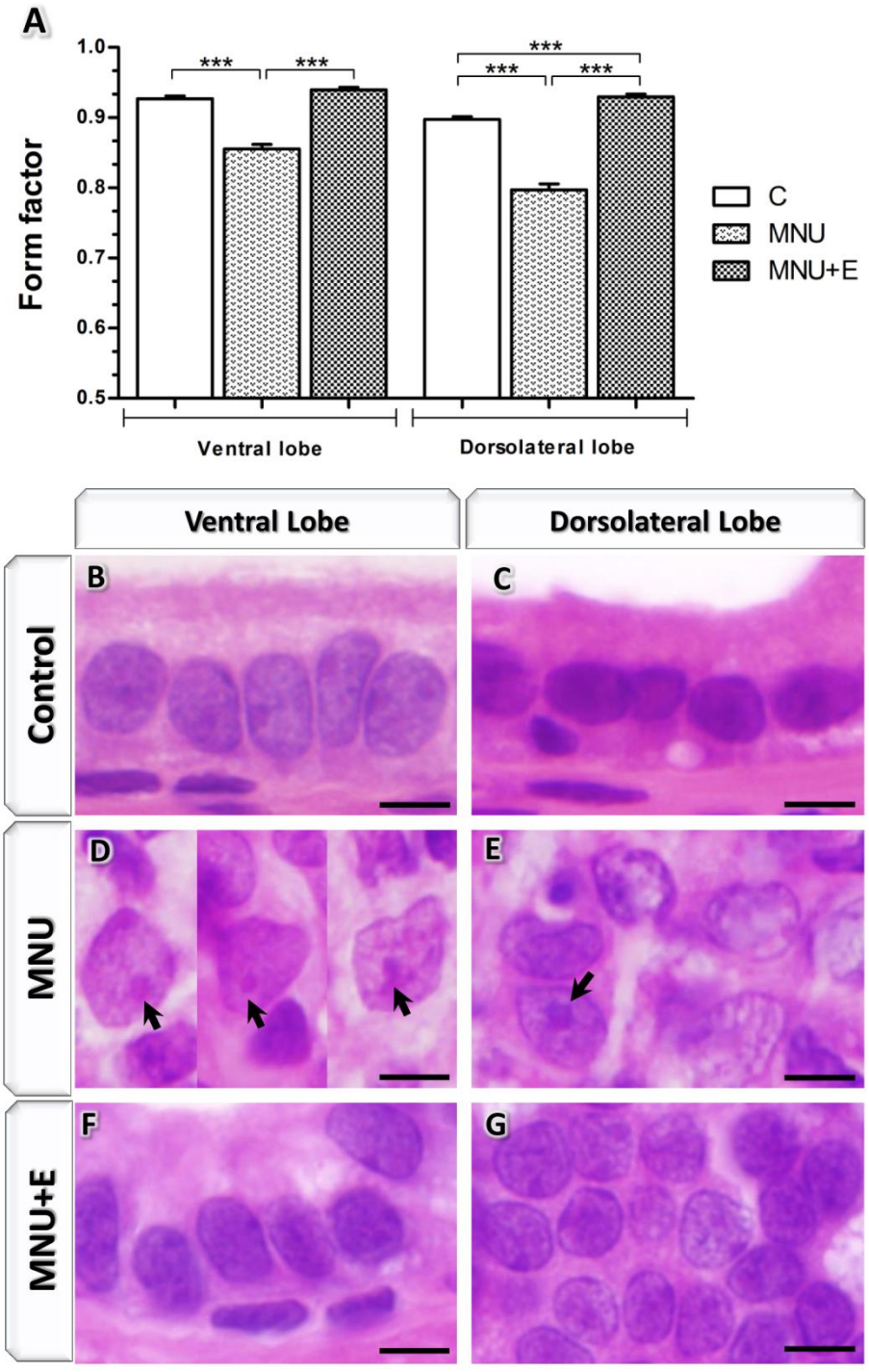

A
Form factor
1.0
0.9
0.8
0.7
0.6
0.5
***
***
***
***
***
C
MNU
MNU+E
Ventral lobe
Dorsolateral lobe
Ventral Lobe
Dorsolateral Lobe
Control
MNU
MNU+E
B
C
D
E
F
G

Figure 6 p63 immunohistochemistry in gerbil ventral and dorsolateral prostate after 28 weeks of treatment. (A) Determination of basal cells (%) in gerbil prostate. Statistical analysis based on ANOVA and Tukey-honest test. Values are represented as mean±SEM (n=5). Superscript (***) indicates P value ≤0.001. (B) Discontinuity of the basal cell layer (*) in a MNU-induced lesions, suggesting invasive properties. Arrows indicates remaining p63 positive cells. (C) Intact basal cell layer in normal epithelium. (D) Increased basal cells in MNU+E group. (E) Intact basal cell layer in a premalignant lesion of MNU+E group. B-E – Ventral lobe. (PIN) Prostate intraepithelial neoplasia. Scale bars = 20μm.

Figure 6.

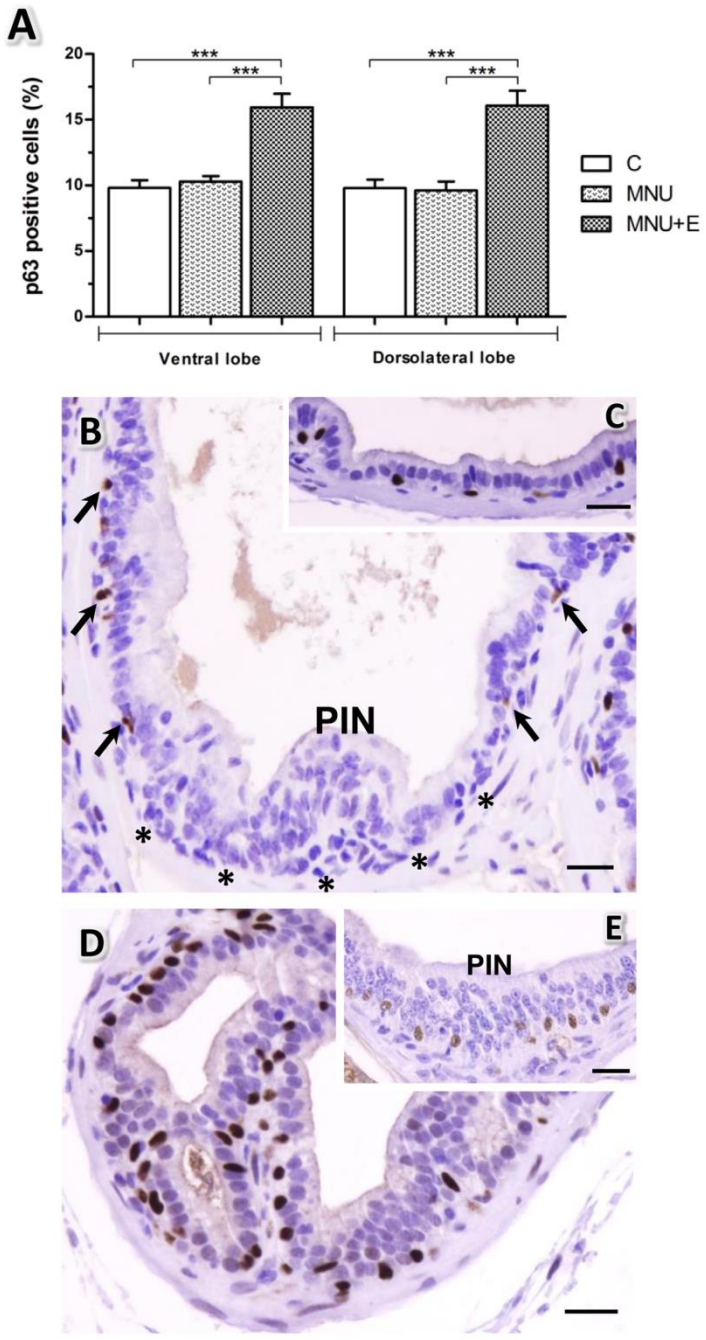

A
p63 positive cells (%)
20
15
10
5
0
***
***
***
***
C
MNU
MNU+E
Ventral lobe
Dorsolateral lobe
B
C
PIN
D
E
PIN

Figure 7 AR immunohistochemistry in gerbil ventral and dorsolateral prostate after 28 weeks of treatment. (A) Determination of AR positive cells (Number of AR-positive cells/field) in gerbil prostate. Statistical analysis based on ANOVA and Tukey-honest test. Values are represented as mean±SEM (n=5). Superscripts indicate statistically significant difference (* P≤0.05; ** P≤0.01; *** P≤0.001). (B-G) Immunolocalization of AR in epithelial compartment. Highest staining proportion was found in epithelial cell nuclei from MNU (D,E) and MNU+E (F,G) groups. Scale bars = 20μm.

Figure 7.

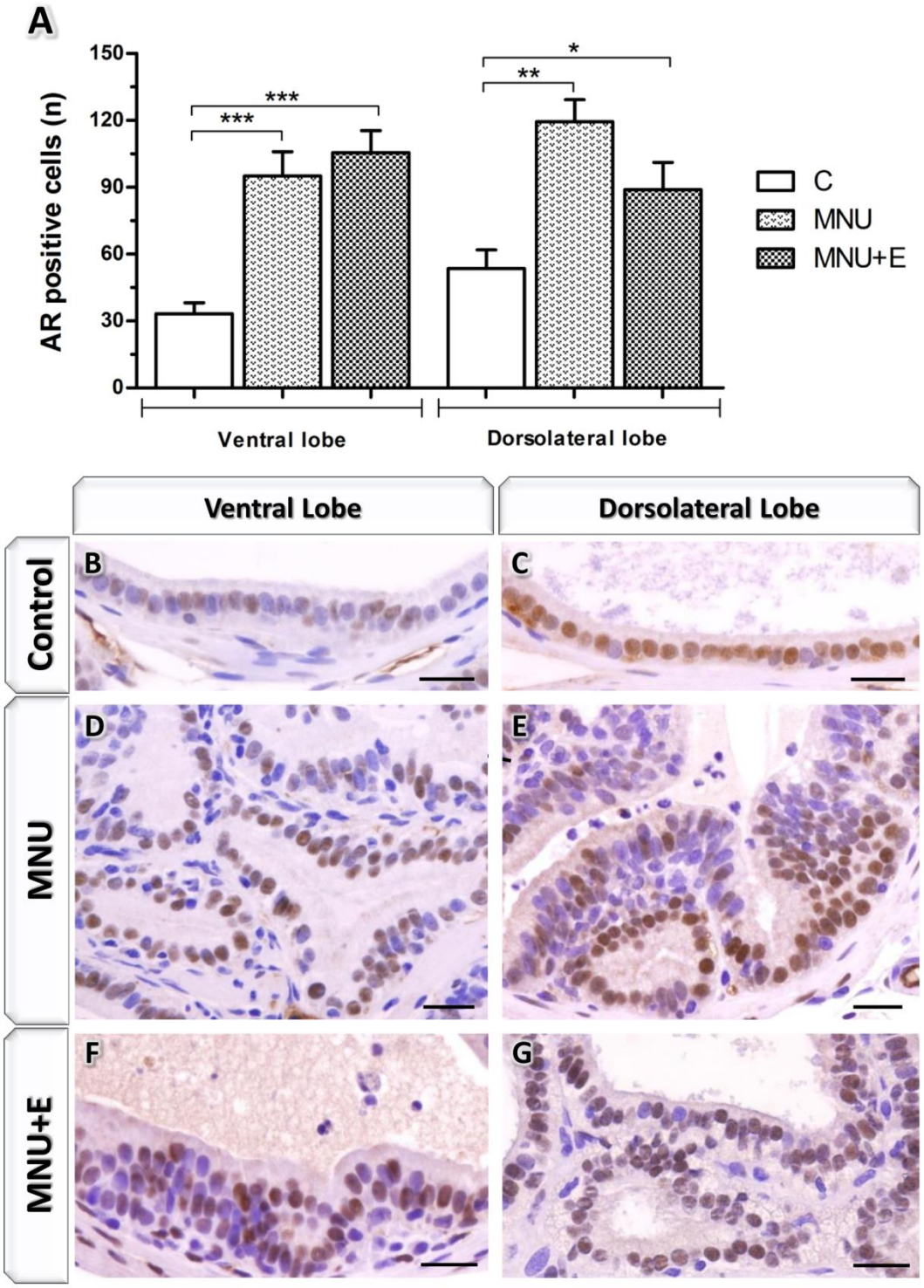

A
AR positive cells (n)
150
120
90
60
30
0
***
***
**
*
C
MNU
MNU+E
Ventral lobe
Dorsolateral lobe
Ventral Lobe
Dorsolateral Lobe
Control
MNU
MNU+E
B
C
D
E
F
G

Figure 8 Global methylation status of gerbil ventral and dorsolateral prostate after 28 weeks of treatment. (A) Determination of labelling index of 5-methylcytidine (percentage of DAB-stained nuclear area over total nuclear area). Values are represented as mean±SEM (n=5). Statistical analysis based on ANOVA and Tukey-honest test. Superscripts indicate statistically significant difference (* $P \leq 0.05$; *** $P \leq 0.01$). (B-G) 5-methylcytidine immunohistochemistry of prostate epithelium Scale bars = 20μm.

Figure 8.

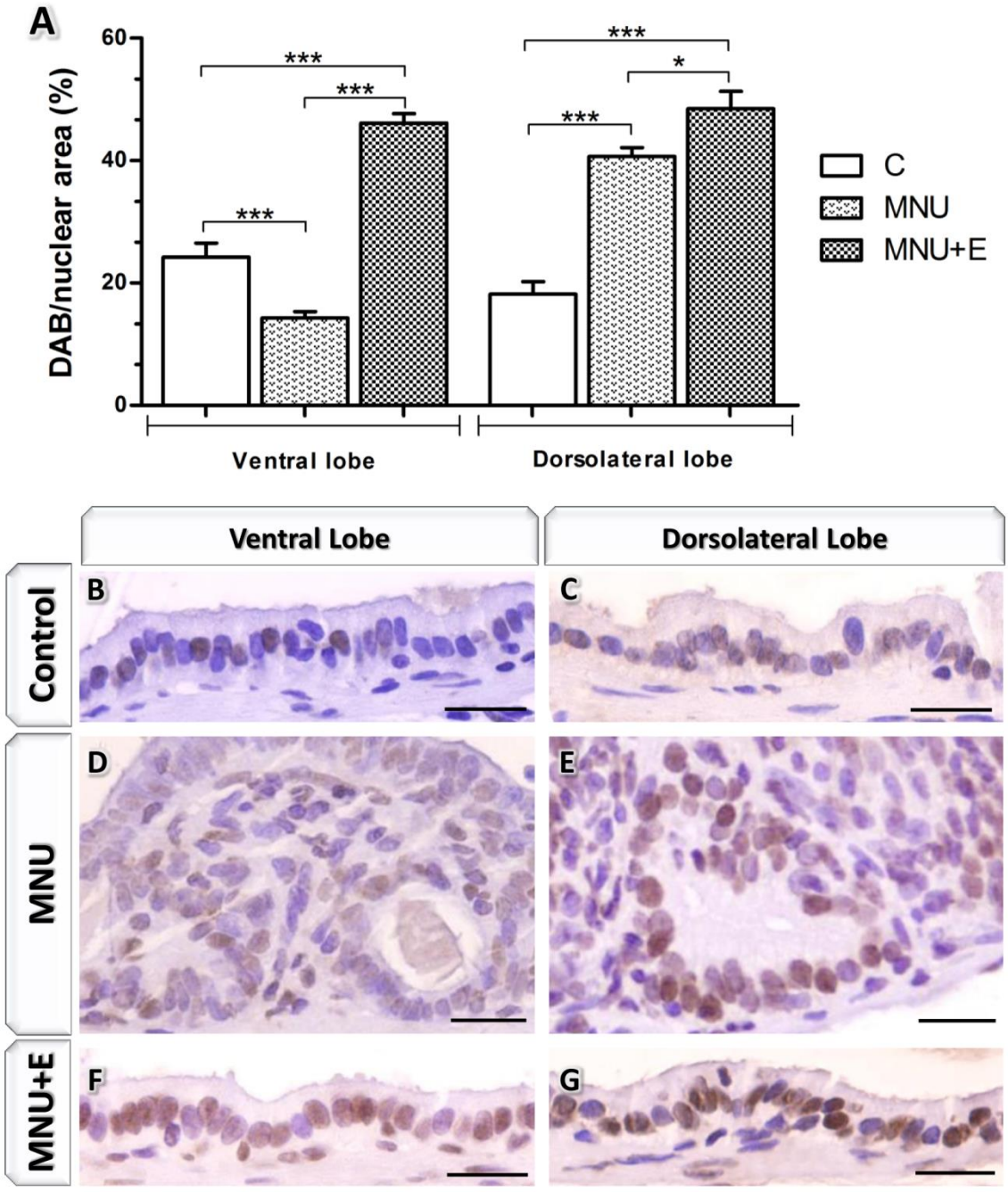
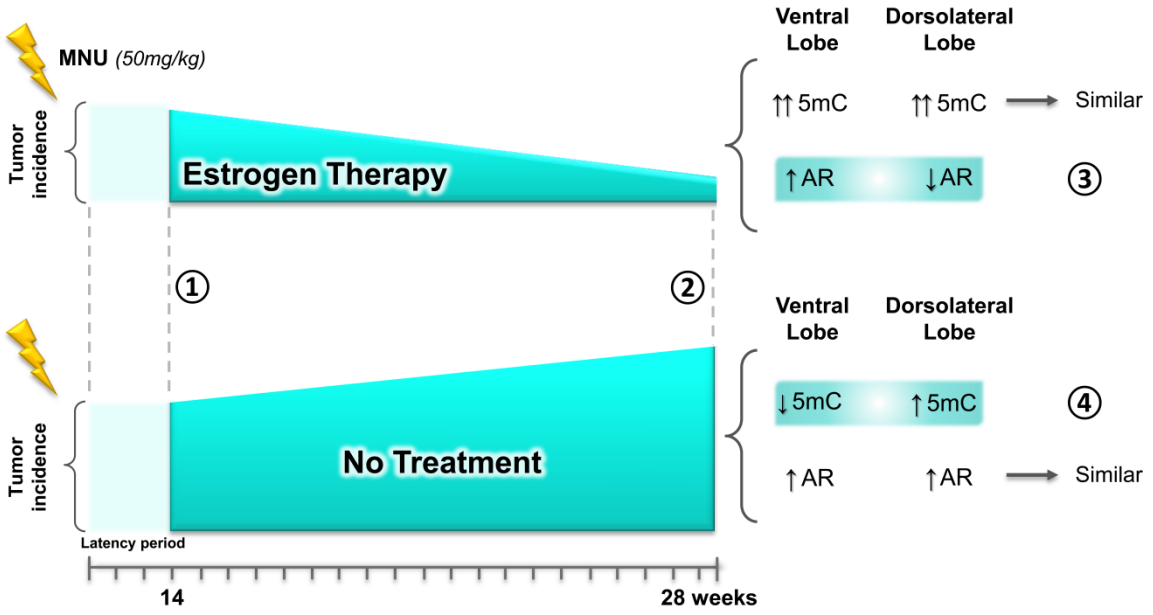

A
DAB/nuclear area (%)
60
40
20
0
***
***
***
***
*
***
C
MNU
MNU+E
Ventral lobe
Dorsolateral lobe
Ventral Lobe
Dorsolateral Lobe
Control
MNU
MNU+E
B
C
D
E
F
G

Figure 9 Schematic representation of histopathological and molecular events that take place during MNU-induced carcinogenesis followed or not by estrogenic therapy. After MNU administration neoplasms develop trough latency period and can be seen in both conditions after 14 weeks ①. After 28 weeks of estrogen treatment, neoplasms growth rate is reduced and some lesions involute and disappear, while tumor growth and progression are maintained without further treatment ②. After 28 weeks, estradiol stimulates epithelium hypermethylation similarly between the lobes. However, the slightly difference in AR positivity possibly explains the neoplastic regression of DL ③. When no further treatment was administered following MNU a similar increase in AR positivity was observed in both lobes. Histopathological differences observed in this situation should not be directly driven by AR but related to the variation of global methylation between lobes ④.

Figure 9.


MNU (50mg/kg)
Tumor incidence
Estrogen Therapy
Ventral Lobe
Dorsolateral Lobe
↑↑ 5mC
↑↑ 5mC
Similar
↑AR
↓AR
③
①
②
Tumor incidence
No Treatment
Ventral Lobe
Dorsolateral Lobe
↓5mC
↑5mC
④
↑AR
↑AR
Similar
Latency period
14
28 weeks

ARTIGO III

EFEITO PROMOTOR DA DIETA HIPERLIPÍDICA SOBRE A CARCINOGÊNESE INDUZIDA POR MNU NA PRÓSTATA VENTRAL DO GERBILO

A ser submetido à revista

"TUMOR BIOLOGY"

EFEITO PROMOTOR DA DIETA HIPERLIPÍDICA SOBRE A CARCINOGÊNESE INDUZIDA POR MNU NA PRÓSTATA VENTRAL DO GERBILO

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Palavras-chave: Câncer de próstata, Gerbilo da Mongólia, N-metil-N-nitrosuréia, Dieta hiperlipídica, MMP-2, *ras*.

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Resumo

Estudos epidemiológicos têm apontado a dieta rica em lipídeos como um fator ambiental potencialmente associado com a progressão tumoral e com as altas taxas de mortalidade por câncer de próstata. Diferentes modelos experimentais têm sido empregados na tentativa de esclarecer o impacto do consumo de dietas ricas em lipídeos sobre a biologia prostática, no entanto os dados permanecem inconclusivos, o que estimula a retomada destas pesquisas empregando novos modelos experimentais ainda não investigados. Assim, o objetivo deste trabalho foi avaliar o potencial promotor da dieta hiperlipídica sobre a carcinogênese induzida por MNU na próstata do gerbilo e caracterizar as neoplasias resultantes dessa associação por métodos histopatológicos, imunohistoquímicos e moleculares. A associação entre MNU e a dieta hiperlipídica promoveu aumento no número de lesões estimuladas pelo carcinógeno isoladamente, as quais exibiram fenótipos característicos de lesões de alto grau. Adicionalmente, as lesões induzidas por essa associação apresentaram maior número de células AR-positivas, pontos de ruptura da camada de células musculares lisas indicando microinvasão tumoral, além da alta reatividade para metaloproteinase do tipo 2. A análise molecular indicou alta expressão das proteínas *ras* em tecidos induzidos por MNU, sugerindo a participação dessa via na promoção e progressão de tumores prostáticos no gerbilo. Desta forma, conclui-se que a dieta hiperlipídica pode ser considerada um agente promotor da carcinogênese prostática, e o gerbilo pode ser empregado como um bom modelo para estudo destas alterações histopatológicas.

Introdução

O câncer de próstata é o terceiro tipo mais comum de câncer diagnosticado em homens e o sexto tipo de câncer mais comum na atualidade (American Institute for Cancer Research & World Cancer Research Fund - AICR &WCRF, 2007). Fatores genéticos e ambientais como origem étnica, história familiar, tabagismo e dieta podem estar envolvidos com a etiologia desta doença (Stacewicz-Sapuntzakis *et al.,* 2008).

Vários estudos epidemiológicos têm apontado a dieta rica em lipídeos como um fator ambiental potencialmente associado com a progressão tumoral e com as altas taxas de mortalidade por cânceres de mama, cólon, pâncreas e próstata em humanos (Fleshner *et al.,* 2004; Kondo *et al.,* 1994). Há um consenso entre estudos correlacionando dietas ricas em gorduras e a patogênese prostática, os quais asseguram que o consumo de ácidos graxos saturados está relacionado com o desenvolvimento do câncer prostático e que ácidos graxos poliinsaturados e gorduras vegetais apresentam uma correlação inversa (Fleshner *et al.,* 2004; Carrol e Khor, 1975; Kolonel *et al.,* 1999).

Diferentes modelos experimentais têm sido empregados na tentativa de esclarecer o impacto do consumo de dietas ricas em lipídeos sobre a biologia prostática. Alguns destes trabalhos correlacionam a ingestão de gordura com o aumento da incidência e da taxa de crescimento de adenocarcinomas prostáticos em ratos Wistar tratados com testosterona (Pollard & Luckert, 1986b), na prole masculina de fêmeas alimentadas com a dieta durante a gravidez (Kondo *et al.,* 1994) e no crescimento tumoral em camundongos TRAMP (transgênicos para adenocarcinoma de próstata) (Park *et al.,* 2013). Entretanto, outros trabalhos falharam em mostrar o papel promotor da dieta hiperlipídica sobre a carcinogênese induzida por tratamento hormonal (Carroll & Noble, 1987) ou pela combinação entre hormônios e carcinógenos químicos (Pour *et al.,* 1991; Shirai *et al.,* 1991) em diferentes linhagens de roedores. Desta forma, os dados permanecem inconclusivos, o que estimula a retomada destas pesquisas empregando novos modelos experimentais ainda não investigados.

Por muitos anos tem-se especulado os mecanismos que determinam a relação entre o consumo de gordura e o risco de câncer de próstata e tem sido determinado que alguns fatores como os níveis hormonais e o estresse oxidativo são afetados pelo tipo de dieta. Consideráveis

evidências têm apontado que alterações nos níveis androgênicos, estimuladas pelo consumo de dietas ricas em gordura, podem elevar o risco de câncer prostático, uma vez que esta doença sofre forte influência hormonal (Fleshner *et al.,* 2004). Níveis hormonais de ratos ACI/Seg que consumiram 5 ou 20% de óleo de milho, correspondendo respectivamente à dieta com baixo e alto teor lipídico, durante longos períodos, indicaram que a testosterona aumentou significativamente e pode ter favorecido o desenvolvimento de neoplasias (Kondo *et al.,* 1994).

A busca pelos mecanismos que relacionam o consumo de dietas ricas em gorduras e o aumento do risco de câncer prostático ainda é objeto de incessantes pesquisas. Desta forma, o emprego de modelos animais, que possibilitem a investigação da dieta como potencial agente promotor da carcinogênese prostática, favorecerá uma melhor compreensão acerca dos mecanismos pelos quais esses macronutrientes modulam a iniciação e progressão do câncer prostático. Assim, o objetivo deste trabalho foi avaliar o potencial promotor da dieta hiperlipídica sobre a carcinogênese induzida por MNU na próstata do gerbilo e caracterizar as neoplasias resultantes dessa associação por métodos histopatológicos, imunohistoquímicos e moleculares.

Material e Métodos

Animais

Foram utilizados 50 gerbilos adultos (100 dias) mantidos no biotério do grupo de pesquisa em *Biologia da Reprodução* do Instituto de Biociências, Letras e Ciências Exatas da UNESP, campus de São José do Rio Preto (SP). Os animais foram mantidos em caixas de polietileno, com substrato de maravalha, em condições controladas de luminosidade e temperatura (23 º C, 40-70% de umidade relativa, 12 claro/12 escuro), alimentados com ração *ad libitum* e água filtrada.

O experimento foi conduzido de acordo com as normas adotadas pelo Colégio Brasileiro de Experimentação Animal (COBEA), sendo aprovado pela Comissão de Ética na Experimentação Animal CEEA/Unesp (Protocolo n.003/2009).

Delineamento Experimental – Indução da Carcinogênese e dietas

Os espécimes foram divididos de maneira aleatória em 3 grupos: **C** (animais controle sem tratamento adicional), **MNU** (dose única de N-metil-N-nitrosouréia – MNU) e **MNU+D** (dose única de MNU e dieta hiperlipídica). Todos os animais foram submetidos à dose única (50mg/Kg) intraperitoneal do carcinógeno MNU (CAS 684-93-5; Sigma, St. Louis, MO). A droga foi estocada a -20ºC e ressuspendida em salina pH 5.5 no momento do uso (0.2 mL/animal) (Bosland e Prinsen, 1990).

Animais do grupo MNU+D foram submetidos à dieta com elevado teor de lipídios e calorias (RC Focus 2413, 2414, 2415, 2416 - Agroceres®), padronizada pelo Laboratório Experimental da Clínica Médica da Faculdade de Medicina de Botucatu-UNESP e utilizada em estudos prévios (Nascimento *et al.,* 2008; Francia-Farje *et al.,* 2010; Ribeiro *et al.,* 2012a,b). Os grupos MNU e controle receberam a ração comercial Labina. A composição das rações controle e hiperlipídica encontram-se na Tabela I. Os quatro tipos de ração hiperlipídica utilizados diferiram apenas no sabor, sendo oferecidas alternadamente ao longo da semana.

Tabela I. Teor de nutrientes das dietas.

Parâmetros	Controle	Hiperlipídica
	Labina	RC Focus 2413, 2414, 2415 e 2416
Proteína (%)	26	20
Carboidrato (%)	54	37
Gordura Saturada (%)	3	20
Calorias (Kcal/g)	3,5	4,8
Outros (%)*	17	23

*Vitaminas, minerais e água

Análise Biométrica e Morfológica

Os animais foram pesados no início e ao final do período experimental para o monitoramento do ganho de peso. Vinte e oito semanas após o inicio do experimento os indivíduos foram mortos por inalação de CO_2, a gordura retroperitoneal e epididimal foi removida e pesada, e foram realizadas medidas da circunferência abdominal. Em seguida o lobo ventral foi excisado e fixado por imersão em Paraformaldeído tamponado ou Metacarn (Metanol, Clorofórmio e Ácido acético, 6:3:1). Os fragmentos foram desidratados em etanol,

clarificados em xilol e, então, incluídos em Paraplast (Histosec™, Merck, Darmstadt, Germany). Cortes seriados de 4μm foram obtidos e corados pela hematoxilina-eosina para estudos morfológicos gerais da próstata e diagnósticos histopatológicos.

Ao longo da série de secções histológicas obtidas foram escolhidos cerca de 25 cortes por grupo experimental para o diagnóstico histopatológico. As lesões histopatológicas detectadas foram classificadas de acordo com o sistema de Classificação Bar Harbor para próstata de camundongos (Shappell *et al.,* 2004) e determinada sua incidência (número de animais acometidos por neoplasias expresso em porcentagem).

Avaliações Imunohistoquímicas

O material foi submetido a técnicas imunohistoquímicas para α-actina de músculo liso (clone IA4, 1:100, Santa Cruz Biotechnology), Receptor de andrógeno (AR – clone N-20, 1:100, Santa Cruz Biotechnology) e metaloproteinase de matriz do tipo 2 (MMP-2 – clone SPM346, pronto para uso, abcam).

Os cortes histológicos desparafinizados e reidratados foram submetidos à recuperação antigênica em tampão citrato pH 6.0 a 97ºC (20-45 min.). O bloqueio de peroxidases endógenas foi efetuado com H_2O_2 (3% em metanol) (20-30 min.) Anticorpos primários para os componentes acima mencionados foram incubados por 60 minutos a 37º ou overnight a 4ºC. Após serem lavados em PBS ou TBS e incubados com sistema de detecção NovoLink Max Polymer (Leica) ou EnVision™+ Dual Link (Dako Cytomation, CA-USA) os cortes passaram pela revelação com a diaminobenzidina (DAB - Dako Cytomation, CA-USA). A contracoloração dos cortes foi feita com hematoxilina de Harris.

Análises Quantitativas

Cortes histológicos aleatórios foram submetidos à imunomarcação para o receptor de andrógeno (22 campos/grupo – aumento de 400x). Para cada campo foi quantificado o número total de células AR-positivas e determinado o número de células marcadas por mm^2 de tecido prostático.

Adicionalmente, fotomicrografias de secções histológicas submetidas a imunomarcação para MMP-2 (15-20 campos/grupo) foram analisadas pelo aplicativo online ImmunoMembrane (Copyright © 2011 Jorma Isola and Vilppu Tuominen - Institute of Biomedical Technology, University of Tampere – http://153.1.200.58:8080/immunomembrane/) e a marcação para cada grupo experimental foi classificada em uma de três categorias. O software ImmunoMembrane usa o principio da deconvolução de cores para separar as marcações da imagem (DAB/Hematoxilina) e um algoritmo customizado para a segmentação da membrana celular (Tuominen *et al.,* 2012). Para a determinação do IM-score são avaliados pelo programa dois componentes: a intensidade de marcação e sua completude. A soma dos dois componentes gera então o IM-score que é considerado para a classificação das amostras nas categorias 0/1+ (de 0 a 5), 2+ (de 5 a 10) ou 3+ (acima de 10).

Análise da Expressão de Proteínas (Western blotting)

Após a remoção do lobo ventral prostático os fragmentos foram mantidos a -80ºC até o início das análises. As amostras foram homogeneizadas mecanicamente a 0ºC em tampão RIPA (Milipore) (0.5M Tris-HCl, 1.5M NaCl, 2,5% deoxycholic acid, 10% NP-40, 10mM EDTA, pH 7,4) contendo inibidores de proteases (Sigma Chemical Co.). O homogeneizado foi centrifugado a 14.000 rpm por 20min a 4ºC, em seguida o sobrenadante foi coletado e submetido à determinação da quantidade de proteínas totais presentes pelo método de Bradford. As alíquotas foram tratadas com solução tampão para corrida de gel (Laemli sample buffer- Bio-rad) e β-mercaptoetanol a 95ºC por 5 minutos sendo empregados 100μg de proteína por amostra. Em seguida, as proteínas foram separadas por eletroforese em SDS-PAGE e transferidas para membranas de nitrocelulose. A ligação inespecífica de proteínas foi bloqueada através da incubação das membranas em leite desnatado 5% em tampão Tris contendo 0.2% de Tween 20 (TBST) por 1 hora em temperatura ambiente. As membranas foram subsequentemente incubadas com o anticorpo primário Anti-pan-*ras* (H-Ras, K-Ras, N-Ras) (F132) e como controle utilizada a β-actina (C4) (Santa Cruz Biotechnology, Califórnia) nas diluições de 1:100-300 em TBST overnight a 4°C. Após lavagem, as membranas foram incubadas em anticorpos secundários especificos (anti-mouse - ab97023; anti-rabbit - ab97051;

Abcam) diluídos 1:10.000 em TBST por 1 hora. Os componentes imunorreativos foram revelados pelo kit de detecção quimioluminescente ECL (GE Healthcare). Análises semi-quantitativas por densitometria das bandas foram realizadas com o auxilio do software Image J (Version 1.33u - National Institutes of Health, USA).

Análise Estatística

Para averiguação da significância entre os dados dos diferentes grupos experimentais, foi empregado o teste estatístico ANOVA seguido do teste de Tukey (para dados biométricos e células AR-positivas) ou do teste de Dunnett's (para análise da expressão de proteínas ras) através do *software* Prism 5.0 (GraphPad). Os dados foram expressos como média±erro padrão e a significância foi determinada para valor de $P \leq 0.05$.

Resultados

Dados Biométricos

O regime de indução tumoral por MNU foi bem tolerado pelos animais, os quais permaneceram saudáveis durante o experimento. Durante o tratamento dos espécimes foi observado que a administração do carcinógeno ocasiona a queda do consumo de ração, principalmente no início do experimento, resultando na queda do peso corporal observada no grupo MNU (Figura 1A,D). No entanto, a ingestão da dieta hiperlipídica, mesmo após a iniciação por MNU, resultou em ganho de peso significativo (Figura 1A,D). Paralelamente, foi verificado grande acúmulo de gordura epididimal (Figura 1B,E) e retroperitoneal (Figura 1B,F) nos animais que ingeriram a dieta hiperlipídica. O acúmulo de gordura corporal, no grupo que recebeu a dieta hiperlipídica, foi concentrado principalmente na região abdominal, elevando significativamente os valores da circunferência da cintura em relação aos demais grupos (Figura 1C).

Análise Histopatológica

A análise histopatológica do lobo ventral da próstata de gerbilos adultos mostrou a alta incidência de lesões pré-malignas espontâneas (Figura 2A). No grupo controle 100% dos animais analisados desenvolveram lesões pré-malignas (Figura 2A). Entretanto, as lesões neste grupo acometeram uma menor proporção da glândula, além de apresentar padrão de crescimento que pode relacioná-las a lesões de baixo grau (Figura 2C). Após a indução com o carcinógeno MNU, as lesões se manifestaram em maior número, ocupando maiores áreas glandulares (Figura 2D). Nos grupos induzidos, as lesões pré-malignas exibiram características indicativas de lesões de alto grau (Figura 2D,F,H). Nestas lesões pode ser observado intenso pleomorfismo nuclear, núcleos atípicos com nucléolos evidentes (Figura 2G). Em particular, após a administração da dieta hiperlipídica, além do aumento da frequência de lesões pré-malignas em relação ao grupo MNU, foi documentada a progressão destas para fenótipos malignos. As lesões malignas foram representadas principalmente por carcinomas microinvasivos e carcinomas *in situ* em menor frequência (Figura 2E).

Análise Imunohistoquímica

A eficácia da metodologia de indução tumoral, na próstata do gerbilo, por MNU associado a agentes promotores, permitiu a investigação de marcadores envolvidos com o processo de tumorigênese na glândula.

Foi verificada a integridade da camada de células musculares lisas que circunda os alvéolos prostáticos (Figura 3A-D). No tecido normal do lobo ventral, esta estrutura se apresenta como uma camada delgada abaixo do epitélio (Figura 3A). Embora animais tratados somente com MNU tenham desenvolvido número considerável de lesões histopatológicas, grande parte destas permaneceram restritas ao interior glandular não havendo sinais de degradação de membrana basal ou ruptura da camada muscular (Figura 3B). Por outro lado as lesões malignas apresentaram células neoplásicas invadindo o estroma glandular adjacente, pela ruptura da membrana basal e da camada muscular lisa dos alvéolos prostáticos (Figura 3C,D), sendo mais frequentemente observadas no grupo MNU+D. Adicionalmente, os focos de

microinvasão neste grupo foram mais frequentes e estruturas semelhantes à ácinos desprovidos de musculatura lisa, característicos de carcinomas *in situ,* foram visualizadas (Figura 3D).

Análise quantitativa da imunorreatividade do receptor de andrógeno e MMP-2

Diante dos dados histopatológicos obtidos, investigamos se o consumo de dieta hiperlipídica afeta marcadores moleculares envolvidos com a carcinogênese, como o receptor de andrógeno e a atividade da MMP-2.

A análise quantitativa do receptor de andrógeno sugeriu a participação da via de sinalização do AR na carcinogênese induzida por MNU (Figura 4A). A associação entre o carcinógeno e o consumo de dieta hiperlipídica elevou marcantemente os níveis de AR no lobo ventral prostático (Figura 4A,D). Em focos histopatológicos foi observada maior frequência de células AR-positivas (Figura 4B,D) em comparação com o epitélio normal (Figura 4C).

Adicionalmente, foi avaliado o padrão de imunorreatividade da MMP-2 no lobo ventral prostático (Figura 5A-E). No grupo controle a quantificação da imunomarcação para a metaloproteinase revelou o índice mais baixo dentre os grupos avaliados, sendo enquadrado na classificação 0/1+ (Figura 5A). No tecido normal a MMP-2 está restrita aos vasos entremeados no estroma glandular e o epitélio se mostra negativo para a reação (Figura 5B). No grupo MNU, a imunomarcação para a protease foi detectada em níveis baixos, porém superiores ao controle, sendo também classificado na categoria 0/1+ (Figura 5A). Por outro lado, a dieta hiperlipídica elevou decisivamente a imunomarcação para MMP-2, que foi classificada como 2+ (Figura 5A). Em particular, a imunorreatividade para MMP-2 foi detectada apenas no epitélio de regiões neoplásicas nos grupos MNU e MNU+D (Figura 5C,D,E). Porém, no grupo MNU+D a imunorreatividade para MMP-2 foi marcadamente intensa (Figura 5D,E), sugenrindo o caráter invasivo das neoplasias.

Expressão de proteínas Ras no tecido prostático

Visando investigar a participação de produtos alterados de genes da família de proto-oncogenes *Ras* (H-ras, K-ras, N-ras) na via carcinogênica estimulada por MNU foi determinada a expressão dessas proteínas por *Western blotting* (Figura 6). A análise da expressão

de proteínas *Ras* no lobo ventral prostático mostrou aumento significativo nos grupos tratados pelo carcinógeno em relação ao controle. Não foi verificada diferença entre os grupos tratados.

Discussão

A predisposição da glândula prostática do gerbilo para o desenvolvimento de lesões histopatológicas tem sido reportada em vários trabalhos do nosso grupo (Campos *et al.,* 2008; Gonçalves *et al.,* 2010; 2013). Lesões pré-malignas espontâneas podem ser observadas em animais adultos com cerca de 6 meses de idade (Gonçalves *et al.,* 2010; 2013), ao passo que aos 18 meses de idade lesões malignas se desenvolvem na glândula (Campos *et al.,* 2008). No entanto, uma redução no período de latência tumoral ocorre quando o animal é exposto a agentes promotores da carcinogênese prostática como a testosterona e sua associação com um iniciador tumoral como o MNU (Pollard & Luckert, 1986a; Gonçalves *et al.,* 2010; 2013). Deste modo, no presente trabalho foi avaliado o potencial promotor da dieta hiperlipídica sobre a carcinogênese induzida por MNU na próstata ventral do gerbilo.

A administração da dieta elevou o peso corporal, resultando em acúmulo significativo de gordura abdominal em relação aos demais grupos. Estes parâmetros, relacionados com a síndrome metabólica, tem sido associados com risco aumentado de câncer prostático (Esposito *et al.,* 2013). No entanto, no período avaliado pelo estudo a indução da obesidade nos animais não foi alcançada. Uma explicação razoável é que a administração do carcinógeno MNU implica na redução de consumo de ração nos dias que sucedem sua aplicação e resulta em ganho de peso lento. Este efeito se deve a sua ação tóxica já verificada em experimentos prévios envolvendo diferentes modelos animais (van Zeeland *et al.,* 2008; Banudevi *et al.,* 2011).

A associação entre o carcinógeno e a dieta hiperlipídica promoveu aumento do número de lesões induzidas pelo MNU isoladamente. Em estudo prévio, empregando modelo similar de dieta hiperlipídica em ratos Wistar, Ribeiro e colaboradores, (2012a) não observaram alterações histológicas marcantes nem lesões malignas na próstata ventral de animais obesos tratados por 15 semanas. As alterações observadas se restringiram a focos hiperplásicos e Neoplasia Intraepitelial prostática (NIP) afetando 0,4% da glândula. Escobar e colaboradores,

(2009) reportaram aumento da frequência de células proliferativas na próstata ventral de animais que receberam dieta rica em gordura saturada por 10 semanas após o desmame, no entanto não demonstraram alterações histopatológicas associadas ao tratamento. Desta forma, o gerbilo demonstra ser um modelo roedor relevante para o estudo das alterações histopatológicas que decorrem do consumo de dietas ricas em lipídeos.

Estudos prévios sugerem que a alteração na ação androgênica na glândula, pode ser um potencial mecanismo pelo qual a dieta promove o desenvolvimento do câncer de próstata (Fleshner *et al.,* 2004). Dietas constituídas por maior proporção de gordura saturada em relação à poliinsaturada elevaram os níveis de andrógenos na urina (Hill *et al.,* 1979) e no plasma de homens (Dorgan *et al.,* 1996), enquanto a redução no consumo mostrou uma relação inversa (Espinosa, 2013). Estes estudos demonstram que a variação no conteúdo de gordura da dieta resulta em oscilações correspondentes nos níveis de andrógenos endógenos, o que tem sido fortemente relacionado ao crescimento tumoral prostático (Akinsete *et al.,* 2012).

Embora no presente estudo os níveis androgênicos não tenham se alterado (dado não mostrado), o aumento de células AR-positivas sugere a participação da via de sinalização do receptor de andrógeno na carcinogênese estimulada pela associação entre MNU e dieta hiperlipídica. A administração de 7% de gordura saturada (proveniente de suínos) a ratos por 10 semanas resultou em aumento dos níveis plasmáticos de testosterona e no aumento da expressão do receptor de andrógeno (Escobar *et al.,* 2009). Contrariamente, dados de Ribeiro *et al.,* (2012a) mostraram redução nos níveis séricos de testosterona e na expressão do receptor de andrógeno. As divergências observadas são provavelmente relacionadas ao estado de obesidade e hiperinsulinemia atingidos no trabalho de Ribeiro e colaboradores, (2012a). Nós especulamos que o MNU isoladamente modula a via androgênica, a qual através do receptor de andrógeno estimula a proliferação celular, e assim contribui para o crescimento tumoral nesse modelo de indução. Outras situações experimentais empregando o mesmo modelo reafirmam nossas constatações, uma vez que evidenciam aumento de células AR-positivas após indução por MNU (Bentel *et al.,* 1999; Liao *et al.,* 2005).

A documentação da progressão tumoral é uma evidência muito importante em modelos experimentais para estudo do câncer prostático (Bostwick, 2000; Shappell *et al.,* 2004). A progressão de lesões pré-malignas para fenótipos malignos pôde ser comprovada pela ruptura da camada de células musculares lisas que circunda os alvéolos prostáticos,

principalmente após a associação do carcinógeno com a dieta hiperlipídica. A progressão de NIP para invasão é provavelmente desencadeada por células cancerosas que produzem enzimas proteolíticas de acordo com a progressão do tumor, o que causa degradação da membrana basal (Liu *et al.,* 2009). De acordo com alguns autores, alterações na composição da membrana basal e outros componentes do tecido conectivo podem influenciar processos como a proliferação e diferenciação da glândula prostática e essas alterações podem ter influência decisiva na etiologia e progressão dos processos patológicos (Tuxhorn *et al.,* 2001).

A MMP-2, uma protease que promove a degradação de vários elementos da matriz extracelular, mostrou intensa imunorreatividade em sítios neoplásicos induzidos pela dieta hiperlipídica. Muitos autores demonstraram uma correlação positiva entre a expressão de metaloproteinases de matriz e o potencial invasivo e metastático de tumores incluindo o câncer retal e gástrico, carcinoma do pulmão, mama, ovário, próstata, câncer da tireoide e tumores cerebrais (Velinov *et al.,* 2010). Células tumorais prostáticas expostas ao soro de ratos obesos sofreram aumento na proliferação, invasão, migração, atividade de metaloproteinases e apresentaram alterações nas proteínas críticas para a transição epitélio-mesenquimal (Price *et al.,* 2012). Assim, a correlação entre o aumento desta protease e as lesões estimuladas pela dieta hiperlipídica, indicam que este agente é capaz de elevar o potencial invasivo de lesões estimuladas por MNU na próstata do gerbilo.

As proteínas Ras exercem papel essencial no controle da ativação de diversas vias de sinalização que regulam a proliferação celular normal. Vários tumores expressam proteínas Ras alteradas, as quais contribuem significativamente para vários aspectos do fenótipo maligno incluindo a desregulação do crescimento tumoral, morte celular programada, invasividade e a indução da angiogênese (Downward, 2003).

Ras tem um papel crucial na integração entre eventos que ocorrem na superfície celular e a transdução de sinal, levando a alteração da transcrição gênica. No câncer prostático, muitos fatores de crescimento e receptores celulares relacionados à via de sinalização de Ras são superexpressos (Zhu & Kyprianou, 2008). A ativação destes receptores inicia a cascata de sinalização Ras/Raf/Mek/Erk que pode levar a ativação do AR via fosforilação ou acetilação, resultando na translocação do receptor para o núcleo e na transcrição de seus genes alvo (Whitaker & Neal, 2010). Dados do presente trabalho mostraram expressão aumentada de proteínas Ras na próstata ventral de animais iniciados pelo MNU, no entanto a administração

da dieta hiperlipídica não interferiu no conteúdo de proteínas ras alteradas após a iniciação pelo carcinógeno. Sukumar e colaboradores (1991) afirmaram que o mecanismo provável de iniciação da carcinogênese prostática por MNU se baseia em uma mutação de ponto no gene *Ki-ras*. Juntamente com os dados histopatológicos e imunohistoquímicos, sugerimos que o MNU afeta a função normal das proteínas Ras, mantendo-as ativas constitutivamente o que pode resultar em ativação da via do AR e de vias adicionais relacionadas à proliferação celular. No entanto, investigações mais acuradas devem ser conduzidas no sentido de esclarecer as alterações moleculares resultantes da administração do carcinógeno sobre a glândula prostática do gerbilo.

Desta forma, conclui-se que o consumo de dietas ricas em lipídeos cria instabilidade na homeostase das células epiteliais prostáticas contribuindo para o estabelecimento e progressão de neoplasias e aquisição de fenótipos malignos. Assim, a dieta hiperlipídica pode ser considerada um agente promotor da carcinogênese no órgão, e o gerbilo pode ser empregado como um bom modelo para estudo destas alterações histopatológicas.

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Legendas das Figuras

Figura 1. Dados biométricos de ganho de peso (A,D), acúmulo de gordura retroperitoneal e epididimal (B,E,F) e circunferência da cintura (C). Os valores representam média±erro padrão (n, 5 por grupo). Análise estatística baseada no teste ANOVA e Tukey-honest. O Super-índice * ($P \leq 0,05$) indica diferenças significativas entre os grupos analisados.

Figura 1.

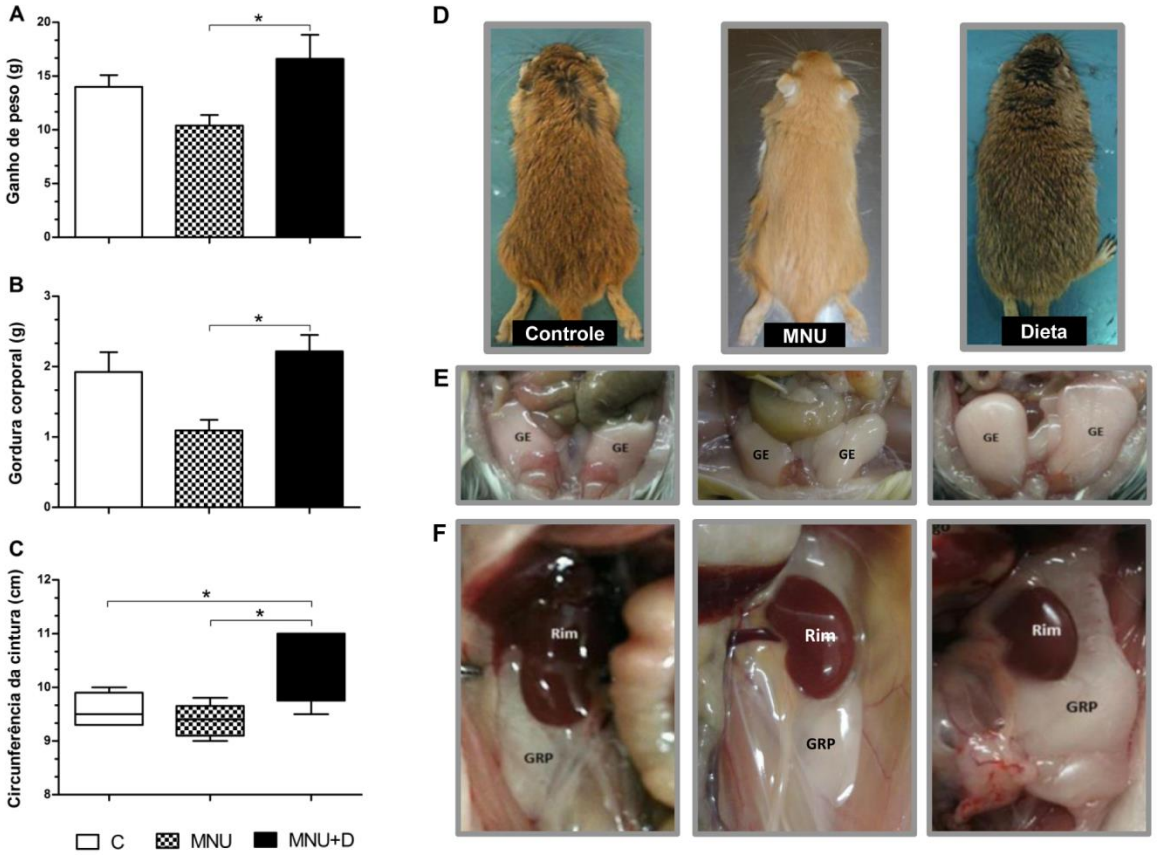

A
Ganho de peso (g)
B
Gordura corporal (g)
C
Circunferência da cintura (cm)
C
MNU
MNU+D
D
Controle
MNU
Dieta
E
GE
F
Rim
GRP

Figura 2. Incidência e caracterização de lesões prostáticas Pré-Malignas e Malignas no lobo ventral da próstata de gerbilos. (A) Os valores representam a porcentagem de animais acometidos por determinado fenótipo neoplásico, sendo consideradas as lesões de maior grau (n, 5 por grupo). (B) Epitélio normal do lobo ventral, (C) NIP de baixo grau no grupo controle, (D) NIP de alto grau no grupo induzido por MNU, (E) Lesão maligna (Carcinoma *in situ*) no grupo MNU+D, (F) Visão geral de região do lobo ventral do grupo MNU+D evidenciando alta frequência de NIP, (G) Pleomorfismo nuclear, núcleos atípicos com nucléolos evidentes (cabeças de seta) em foco neoplásico do grupo MNU+D, (H) Detalhe da região destacada em *F* mostrando NIP com padrão de crescimento cribiforme. Ep – Epitélio, ML – Músculo liso, NIP – Neoplasia Intraepitelial Prostática, * – Vasos sanguíneos.

Figura 2.

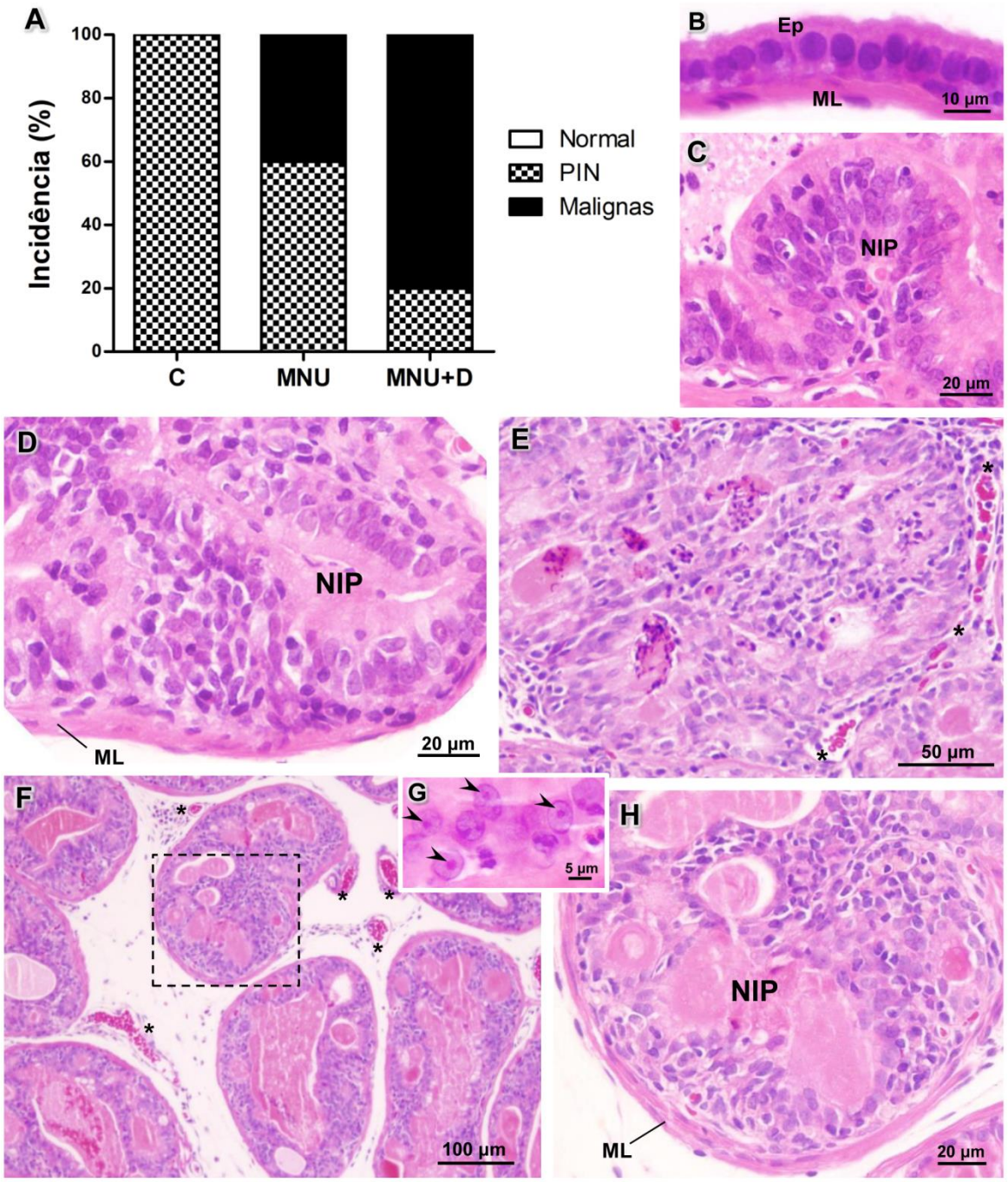

A
Incidência (%)
100
80
60
40
20
0
C
MNU
MNU+D
Normal
PIN
Malignas
B
Ep
ML
10 µm
C
NIP
20 µm
D
NIP
ML
20 µm
E
50 µm
F
100 µm
G
5 µm
H
NIP
ML
20 µm

Figura 3. Imunohistoquímica para α-actina de músculo liso. (A) Tecido normal do lobo ventral exibindo a camada delgada de músculo liso abaixo do epitélio, (B) Lesão pré-maligna (NIP) que retém a camada de músculo liso no lobo ventral do grupo MNU, (C) Ruptura na camada de músculo liso (setas) que envolve o alvéolo permitindo a invasão estromal por células neoplásicas (*) de carcinoma microinvasivo no lobo ventral do grupo MNU+D, (D) Foco amplo de carcinomas *in situ* desprovido de musculatura lisa, no centro da imagem. Setas – Rupturas na camada de músculo liso, L – Lúmen, ML – Músculo liso, NIP – Neoplasia Intraepitelial Prostática, * – Focos microinvasivos.

Figura 3.

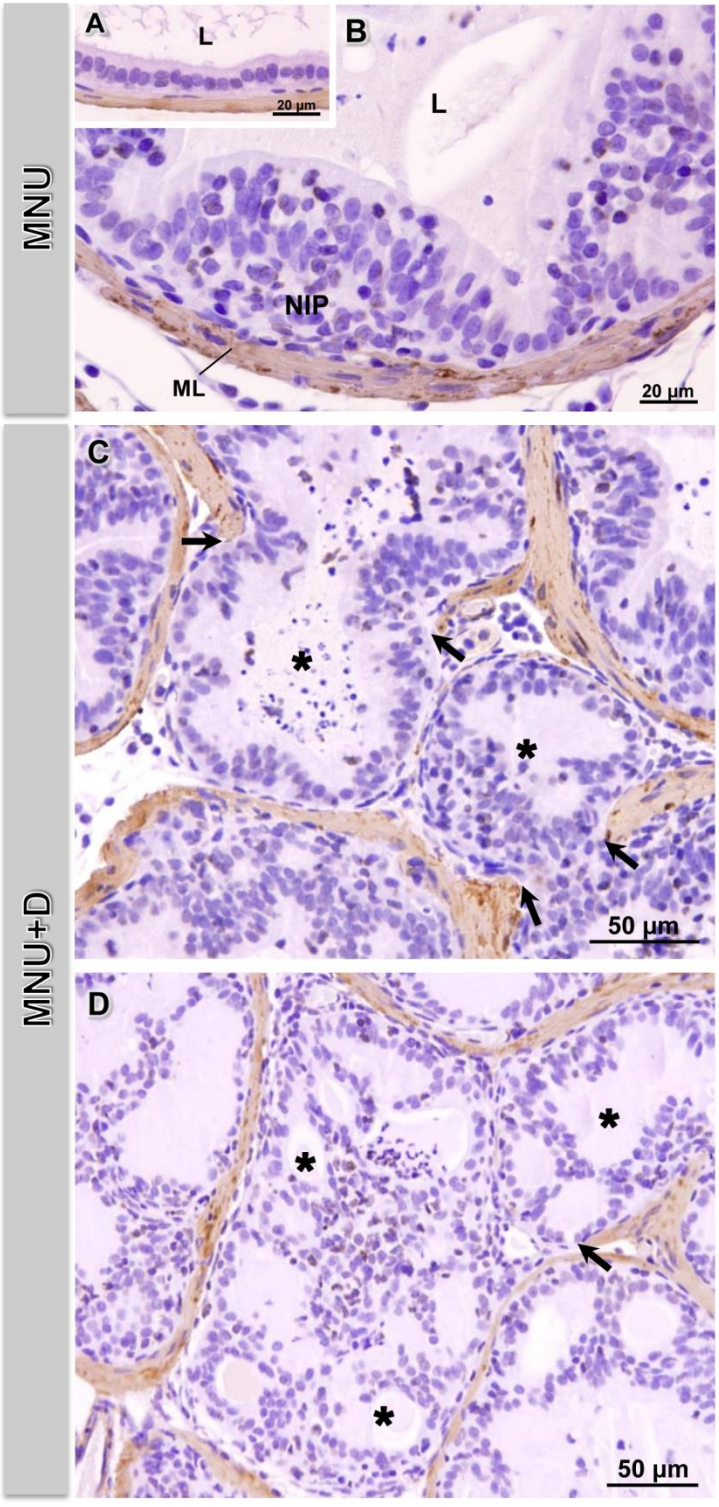


Figura 4. Imunolocalização do receptor de andrógeno no lobo ventral da próstata de gerbilos. (A) Determinação de células AR-positivas por mm^2. Os valores representam média±erro padrão. Análise estatística baseada no teste ANOVA e Tukey-honest. Super-índices (* P≤0,05; *** P≤0,001) indicam diferenças significativas entre os grupos analisados. (B) Células AR-positivas em neoplasia do grupo MNU, (C) Epitélio normal do grupo C mostrando células AR-positivas, (D) Aumento do número de células AR-positivas em NIP cribiforme do grupo MNU+D.

Figura 4.

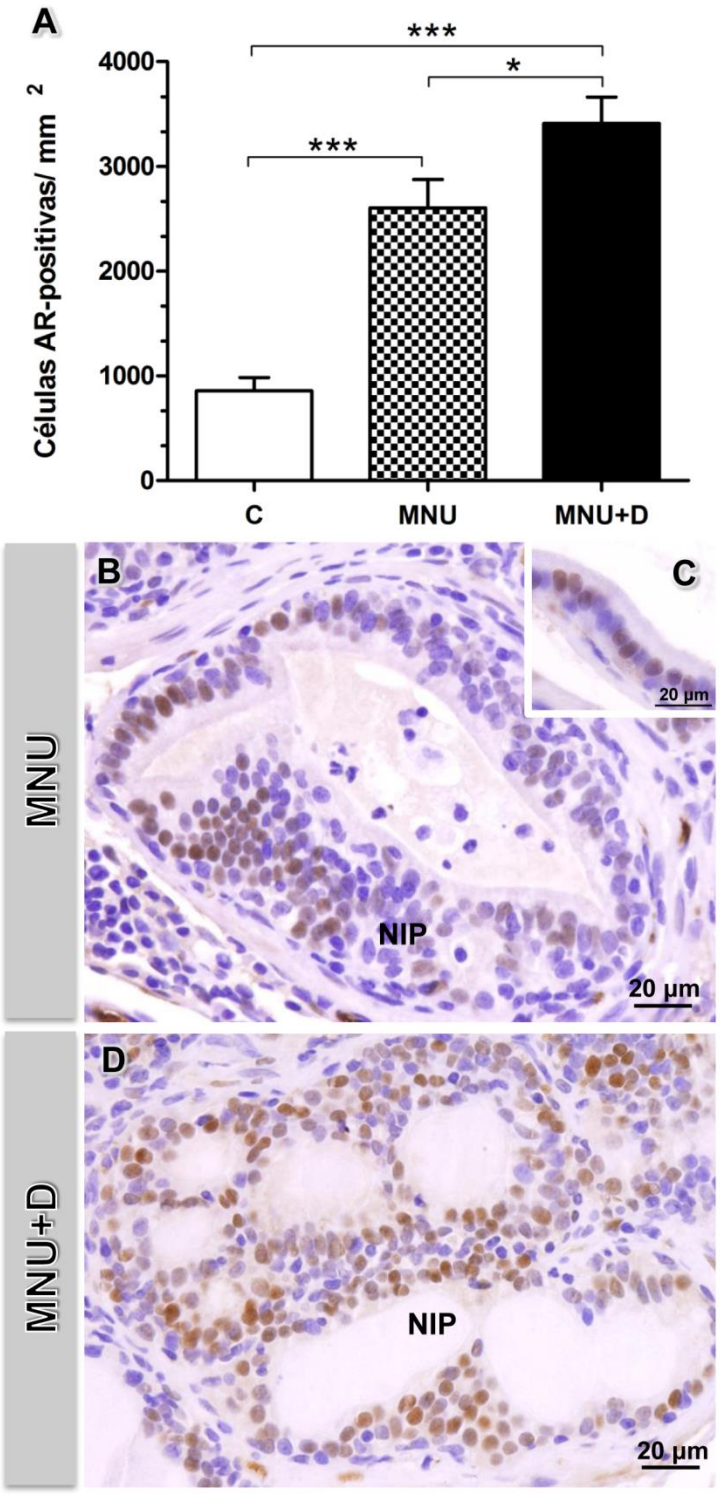

A
Células AR-positivas/ mm 2
4000
3000
2000
1000
0
***
*
***
C
MNU
MNU+D
B
C
20 µm
MNU
NIP
20 µm
D
MNU+D
NIP
20 µm

Figura 5. Imunorreatividade do lobo ventral a MMP-2. (A) IM-score e classificação do padrão de imunomarcação da MMP-2 no diferentes grupos experimentais. (B) O epitélio normal se mostra negativo para MMP-2, a qual se restringe aos vasos entremeados no estroma glandular (seta), (C) No grupo MNU baixa imunorreatividade para MMP-2 foi detectada no epitélio neoplásico, (D e E) A administração da dieta hiperlipídica elevou imunomarcação da MMP-2 no epitélio de áreas neoplásicas. NIP – Neoplasia Intraepitelial Prostática, NIP* – Lesão com projeção de células neoplásicas para o estroma glandular.

Figura 5.

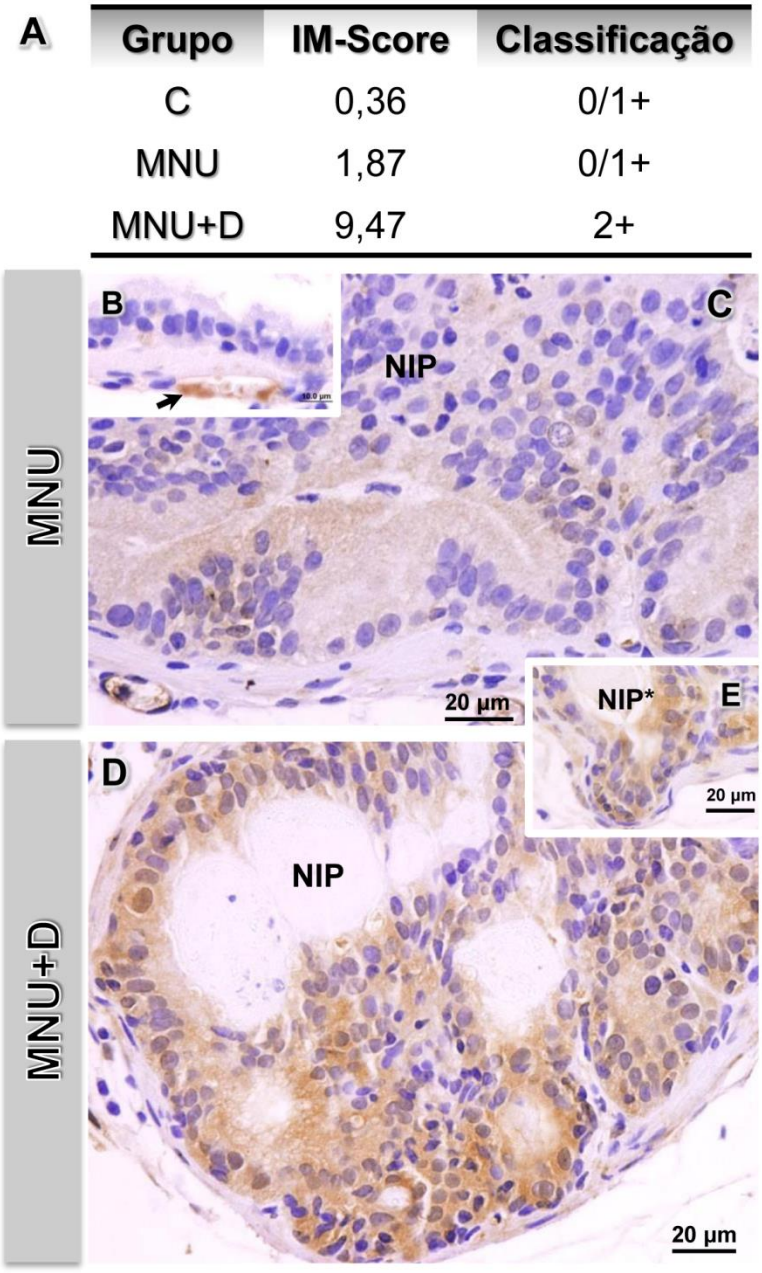
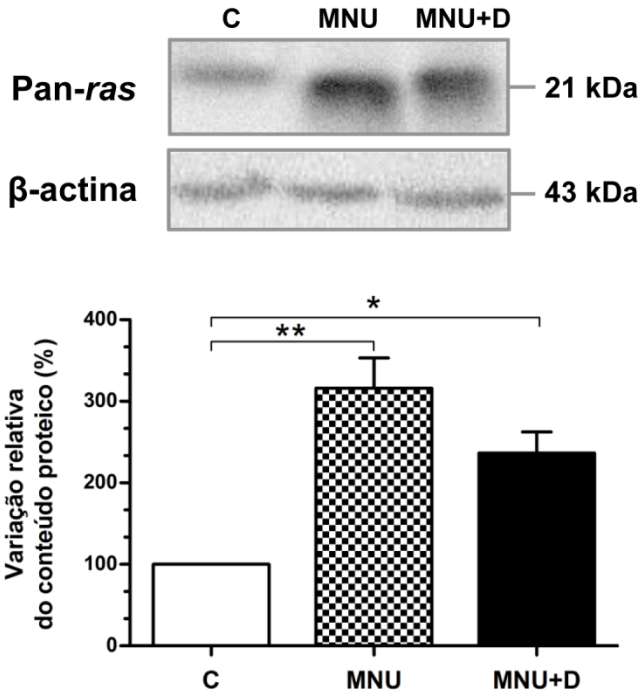

A
Grupo
IM-Score
Classificação
C
0,36
0/1+
MNU
1,87
0/1+
MNU+D
9,47
2+
MNU
MNU+D
B
C
NIP
20 µm
NIP*
E
20 µm
D
NIP
20 µm

Figura 6. Análise molecular de proteínas Ras (*Western blotting*). Variação relativa do conteúdo proteico entre os grupos experimentais analisados (expresso em % em relação ao grupo Controle). β-actina foi considerada como controle constitutivo da reação. Os valores representam média±erro padrão. Análise estatística baseada no teste ANOVA seguido do teste de Dunnett's. Super-índices (* P≤0,05; ** P≤0,01) indicam diferenças significativas entre os grupos experimentais em relação ao controle.

Figura 6.


C
MNU
MNU+D
Pan-*ras*
21 kDa
β-actina
43 kDa
Variação relativa
do conteúdo proteico (%)
400
300
200
100
0
*
**
C
MNU
MNU+D

CONCLUSÕES

- ✓ O gerbilo pode ser incluso como um bom modelo para investigação do comportamento morfofisiológico e patológico da glândula prostática, uma vez que desenvolve lesões pré-malignas espontâneas, as quais aumentam em número após tratamento com o carcinógeno N-metil-N-nitrosuréia;

- ✓ A indução tumoral baseada na associação entre MNU e testosterona estimula o aparecimento das primeiras lesões proliferativas após 3 meses de tratamento. Após 6 meses as neoplasias progridem e atingem números estatisticamente relevantes. Assim, este modelo apresenta latência tumoral inferior quando comparado aos modelos roedores baseados em protocolos similares de indução tumoral;

- ✓ A iniciação e progressão tumoral ocorrem de maneira diferencial nos lobos ventral e dorsolateral da próstata do gerbilo, o que sugere a participação de vias de sinalização distintas durante o curso da carcinogênese em diferentes regiões do órgão;

- ✓ A prolongada exposição a altas doses de estrógeno preveniu o aparecimento e reduziu a taxa de crescimento de lesões induzidas por MNU apresentando, portanto um efeito terapêutico. Entretanto, o epitélio remanescente apresentou características distintas de um epitélio normal, as quais podem favorecer a recidiva das neoplasias em período posterior ao analisado;

- ✓ O padrão de metilação global do DNA difere entre os lobos prostáticos do gerbilo após tratamento com o carcinógeno. Este fator é então sugerido como um dos responsáveis pelas diferenças observadas entre as regiões da glândula prostática com relação ao desenvolvimento de neoplasias;

- ✓ A associação entre o carcinógeno e a dieta hiperlipídica elevou a incidência de lesões histopatológicas, as quais expressaram marcadores que contribuem para a invasividade

tumoral. Assim, a dieta hiperlipídica pode ser considerada um agente promotor da carcinogênese prostática;

- ✓ As neoplasias induzidas por MNU na próstata do gerbilo apresentam alta expressão de proteínas Ras, sugerindo a participação dessa via na promoção e progressão dos tumores prostáticos.

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APÊNDICE

KEY PARTICIPANTS OF THE TUMOR MICROENVIRONMENT OF THE PROSTATE: AN APPROACH OF THE STRUCTURAL DYNAMIC OF CELLULAR ELEMENTS AND EXTRACELLULAR MATRIX COMPONENTS DURING EPITHELIAL-STROMAL TRANSITION

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ABSTRACT

Cancer is a multistep process that begins with the transformation of normal epithelial cells and continues with tumor growth, stromal invasion and metastasis. The remodeling of the peritumoral environment is decisive for the onset of tumor invasiveness. This event is dependent of epithelial-stromal interactions, degradation of extracellular matrix components and reorganization of fibrillar components. Our research group has studied in a new proposed rodent model the participation of cellular and molecular components in the prostate microenvironment that contributes to cancer progression. Our group adopted the gerbil *Meriones unguiculatus* as an alternative experimental model for prostate cancer study. This model has presented significant responses to hormonal treatments and to development of spontaneous and induced neoplasias. The data obtained indicate reorganization of type I collagen fibers and reticular fibers, synthesis of new components such as tenascin and proteoglycans, degradation of basement membrane components and elastic fibers and increased expression of metalloproteinases. Fibroblasts that border the region, apparently participates of the stromal reaction. The roles of each of these events as well as some signaling molecules participants of neoplastic progression and factors that promote genetic reprogramming during epithelial-stromal transition are also discussed.

Keywords

Extracellular matrix, prostate cancer, stromal remodeling, rodent model.

OVERVIEW

Cancer is a multistep process that begins with the transformation of normal epithelial cells and continues with tumor growth, stromal invasion and metastasis. In humans, prostate cancer has a prolonged natural history that can be conceptually divided into three stages. The first and most prolonged stage involves the initiation and development of organ-confined disease, estimated to take more than 15 years. The second stage involves spreading into regional lymph nodes, and the third stage involves metastatic dissemination via both the lymphatic and blood circulations to the skeleton (Johansson *et al.* 1997).

Malignant progression in cancer requires populations of tumor-initiating cells endowed with unlimited self-renewal, survival under stress, and establishment of distant metastases. Additionally, the acquisition of invasive properties driven by epithelial- mesenchymal transition (EMT) is critical for the evolution of neoplastic cells into fully metastatic populations (Celià-Terrassa *et al.* 2012). EMT consists in a transdifferentiation process of differentiated epithelial cells that acquire fibroblastoid-like phenotypes. Although this process occurs during embryogenesis and wound healing, pathological EMT is increasingly recognized to play an important role during the development of human diseases, such as chronic inflammation, fibrosis, rheumatoid arthritis, and cancer invasion and metastasis (Micalizzi *et al.* 2010; Taylor *et al.* 2010; Thiery *et al.* 2009). Phenotypic and morphologic alterations that affect epithelial cells during EMT cooperate to transform immobile multicellular epithelial sheets into highly motile, independent cells that are able to invasion and metastasis (Wendt *et al.* 2012) and endowed with self-renewal and tumor-initiating capabilities coupled to chemoresistance (Wendt *et al.* 2012).

Many of the studies involving spontaneous or chemically induced prostate cancer until recently focused on the behavior of anomalous epithelial cells, which are the main targets for molecular and chemoprevention studies. However, it is becoming clearer that the progression of malignant tumors is not exclusively regulated by the disruption of the oncogene action pathway and tumor suppressor genes in neoplastic cells. Much evidence suggests that tumor progression co-depends on stromal behavior for creating a micro environment that promotes tumorigenesis (Tuxhorn *et al.* 2001; Hanahan and Weinberg 2011). Several classes of novel therapies that disrupt signaling pathways within tumor microenvironments are currently

under investigation. In contrast to cytotoxic therapies that principally target the epithelial cell (regardless of its anatomic location), these agents disrupt the "crosstalk" between epithelial cells, stromal cells, and the extracellular matrix (ECM) necessary for prostate cancer progression and metastasis (Corn, 2012).

Increasing evidence supports the importance of epithelial–stromal cell interaction in tumor growth, progression, angiogenesis, and therapeutic resistance. Moreover, interactions between stromal and epithelial cells result in reciprocal influences. Despite the importance of these interactions in tumorigenesis, the underlying molecular mechanisms are poorly characterized, in part because of their complexity and redundancy (Hu and Polyak 2008; Hu *et al.* 2009).

Prostatic stromal compartment is composed of cells such as fibroblast, myofibroblasts and smooth muscle cells which secrete growth factors, produce ECM, and express androgen receptors, estrogen receptors, adrenergic receptors and 5-α reductase (Berry *et al.* 2008). The ECM includes a three dimensional fibrillar meshwork of interstitial tissues and two dimensional sheets of basement membrane (BM), which underlies epithelial and endothelial tissues (LeBleu *et al.* 2007; Rowe and Weiss 2009). Structural components, such as collagen and elastic fibers, provide mechanical strength and flexibility to tissue and also serve as a substrate for cell attachment and migration, which are mediated by adhesive glycoproteins, such as fibronectin and laminin. Moreover, the structure and permeability of ECM are regulated by proteoglycans, which also bind and modulate the activity of growth factors, proteases and protease inhibitors (Tuxhorn *et al.* 2001).

Tumor initiation and progression are determined by molecular and phenotypic alterations arising in the tumor epithelial cells as well as in their microenvironment (Hu and Polyak 2008). Epithelial cells have been proposed as a source of reactive stroma via EMT (Zavadil *et al.* 2008, Kalluri 2009). Several molecules are involved in inducing EMT, including TGF-β, interleukin-8, fibroblast growth factors, connective tissue growth factor, stromal cell-derived factor-1, among others. In general**,** these factors have the potential to reach and regulate the stromal compartment when the epithelial layer's integrity is disrupted during cancer progression (Barron and Rowley 2012). The reactive stroma is characterized by: phenotypic alterations in stromal cells, increase in angiogenesis, influx of macrophages associated with the tumor, as well as the remodeling of the ECM and availability of paracrine

factors and proteases (Figure 1) (Tuxhorn *et al.* 2001: Bhowmick *et al.* 2005). In this scenario studies show that myofibroblasts are detectable in the tissues surrounding the foci of prostate intraepithelial neoplasia, and their activity increases with increasing prostate tumor grade (Tlsty and Coussens 2005; Tuxhorn *et al.* 2002).

TGF-β has been extensively studied since it regulates numerous biological processes involved with prostate homeostasis (Jones *et al.* 2009; Barron and Rowley 2012) and shows increased expression in prostate cancer (Ao *et al.* 2007, Alonso-Magdalena *et al.* 2009). In normal conditions TGF-β acts as a tumor suppressor and during EMT it acquires the properties to induce metastatic progression (Wendt *et al.* 2012). Among the pathways responsible for these actions Ao *et al.* 2006 pointed to constitutively activation of Akt, which functioned to inhibit Smad3 and p21 translocation to the nucleus avoiding the apoptotic effects of TGF-β signaling**.** VEGF/VEGF receptor, FGF/FGF receptor, platelet-derived growth factor/platelet-derived growth factor receptor are angiogenic factors often released by reactive stroma (Corn 2012).

The vast heterogeneity of prostate tumors at the molecular and pathological levels has demanded efforts for establishing animal models that mimic the initial steps of prostate cancer, as well as its behavior, progression and metastasis. Each model is characterized by unique features contributing to the understanding of prostate tumorigenesis, as well as limitations challenging our knowledge of the mechanisms of cancer development and progression (Hensley and Kyprianou 2012).

Our research group adopted the gerbil *Meriones unguiculatus* as an alternative experimental model for prostate cancer study. This model has been providing a good alternative to study the alterations that occur in the stromal microenvironment of the prostate gland during carcinogenesis, since it develops spontaneous neoplasia (Pegorin de Campos *et al.* 2006; Campos *et al.* 2008; Custódio *et al.* 2008) and induced neoplasia (Gonçalves *et al.* 2010, 2013) in a relevant number of individuals, presenting significant responses regarding hormonal treatment (Santos *et al.* 2006; Scarano *et al.* 2006; Oliveira *et al.* 2007; Scarano *et al.* 2008).

The prostatic complex of the male gerbil is formed by four pairs of lobes closed associated with the urethra, i.e, anterior lobes (or coagulating gland), ventral lobe, dorsal lobe and dorsolateral lobe and each prostate lobe is enclosed by a delicate capsule of mesothelial cells. Dorsolateral lobe and ventral lobe present similar relative volumes between epithelial and

stromal compartments, together occupying approximately 40% of the total organ (Rochel *et al.* 2007). Dorsolateral lobe is the most voluminous component of the gerbil, which differs from other rodents such as the rat and mouse, where the dorsal lobe is the least developed and the ventral lobe corresponds to 50% of the total volume of the whole prostatic complex (Hayashi *et al.* 1991). Anterior lobe, ventral lobe and dorsolateral lobe are composed of ductal glands without true acinar components, and the dorsal lobe is the only one of these to show tubule–acinar organization (Rochel *et al.* 2007). Additionally, previous data from our laboratory have demonstrated that histological, histochemical, and ultrastructural features of the adult gerbil prostate are comparable with those of the human prostate, such as the smooth muscle cell structure and ultrastructure (Corradi *et al.* 2004) and epithelium cell types (Santos *et al.* 2003; Pegorin de Campos *et al.* 2006).

Another important feature of gerbil is that the female also presents a prostate gland (Santos *et al.* 2003; Custódio *et al.* 2004) and it is homologous with the ventral prostate in male rodents (Gross *et al.* 1987; Corradi *et al.* 2004). While the male prostate surrounds the urethra, the female prostate lies in the wall of the female urethra (Zaviačič 1999; Custódio *et al.* 2004). This is the basic macroscopic difference between the male and female prostate glands. Despite the smaller space available for the female prostate surrounding the female urethra, it possesses all the structural components characteristic of the male prostate, such as functional epithelium and developed surrounding stroma (Zaviačič 1999; Santos *et al.* 2008).

The development of spontaneous prostate lesions is an interesting feature of this rodent species and these are more frequent in the dorsolateral and ventral lobes. At 18 months of age, some degree of prostate lesion was found in 46% of the animals analysed by Campos *et al.* (2008). The most frequent epithelial alterations were the PINs, which appeared in 46.67% (14⁄30) of the analyzed prostates and microinvasive lesions were less representative, 26.67% (8/30). After administration of testosterone isolated or in association with MNU, adult animals (90 day) developed lesions at 6 months. Malignant lesions, characterized by carcinomas and adenocarcinomas, have increased during the treatment time (Gonçalves *et al.* 2010). Comparisons between dorsolateral and ventral lobes revealed that lesions developed first in the dorsolateral while the ventral lobe presented longer tumor latency. However, after 6 months, there was a dramatic increase in tumor multiplicity in the ventral lobe mainly in MNU+Testosterone treated group. This study also showed that there are distinct pathways

involved in tumor progression in gerbil prostate lobes (Gonçalves *et al.* 2013). The increase in lesions during treatment were also observed by Scarano *et al.* (2006) in aged gerbil treated with testosterone, but the authors also believe that the androgen promoted the reversion of the natural effects of aging on the prostate in some animals. On the other hand, different types of hormonal ablation (orchiectomy, cyproterone acetate, flutamide and/or tamoxifen administration or blockade of the steroid metabolizing enzymes) at different ages showed that this rodent model is also extremely dependent on serum levels of testosterone (Corradi *et al.* 2004, 2009; Oliveira *et al.* 2007; Cordeiro *et al.* 2008; Campos *et al.* 2010, 2011). Just after hormone replacement, the recovery of morphology and prostatic functions could be observed (Oliveira *et al.* 2007; Cordeiro *et al.* 2008).

For this large amount of information about gerbil prostate and its versatility in responding to different experimental conditions and being susceptible to neoplastic lesion development we believe that it is an important model for understanding the biology and histopathology of this gland.

This review discusses the behavior of some traditional ECM proteins, Collagen I, III and IV, laminin, elastin as well as matricellular components such as tenascin, proteoglycans and matrix metalloproteinases in prostate cancer initiation and progression, with special attention to our animal model, besides some growth factors involved with EMT process which also favors cancer progression and metastatic events.

EXTRACELLULAR MATRIX FIBRILLAR ELEMENTS

Collagen Type I and III

Collagen I is a heterotrimeric, fibrillar protein that is the major ECM component produced by myofibroblasts (van Hoorde *et al.* 2000; Chung *et al.* 2005; Gordon and Hahn 2010). Collagen fibers not only function as a scaffold for the tissue but also regulate the expression of genes associated with cellular signaling and metabolism, and gene transcription and translation (Morrison *et al.* 2000; van Hoorde *et al.* 2000). Thus, it affects fundamental cellular processes that are essential for tumor progression, such as cell survival, apoptosis and

cell invasion (Cheng and Leung 2011). ECM in general, and collagen type I in particular, can promote EMT, which is an additional source of myofibroblasts.

In normal conditions, the prostate reveals collagenous fibers of different thicknesses and with different directions creating a three-dimensional network (Taboga and Vidal 2003). In the prostatic stroma of gerbils, the network of collagenous fibers is loose and it presents regularity in spatial organization, observed as a dense layer under the acinar epithelium (Campos *et al.* 2008; Custódio *et al.* 2008; Gonçalves *et al.* 2010). In conditions of tissue homeostasis, the collagen fibrils present a slow metabolic turnover; however, under conditions of tissue remodeling, this process is increased. Various studies have suggested the cleavage of type I collagen as an integral component of neoplastic stroma in the structural as well as the signaling role, since proteolysis leads to the formation of biologically active collagen I peptides, which favor the proliferation and angiogenesis, while also representing a therapeutic target (Seandel *et al.* 2001; Tlsty and Coussens, 2005). Moreover, it has been shown that collagen I can induce EMT in various types of epithelial cancer cells and is highly expressed in metastatic tumors. Collagen I increases cell invasiveness by reducing E-cadherin-mediated cell–cell adhesion and the loss of E-cadherin is the key feature of the EMT. Down-regulation of E-cadherin is mainly due to the up-regulation of Snail, Slug, Twist, ZEB1 and other transcription factors, which is mediated by collagen I, and results in the repression of E-cadherin (Cheng and Leung 2011).

In Gleason 7 adenocarcinomas (Tuxhorn *et al.* 2001) and in spontaneous and induced microinvasive carcinomas in the gerbil prostate, collagen I fibers were intensely fragmented, with a large diameter, disperse arrangement and reduction of fibers inside the cellular aggregate (Figure 2a,b) (Campos *et al.* 2008; Gonçalves *et al.* 2010). Taboga and Vidal (2003) proved that the progression of tumor malignancy is accompanied by the formation of a scaffold of fine and branched collagen fibers. In our model it is also possible to observe that the epithelial cell in the migratory process interacts with type I collagen fibers (Figure 4a). There is evidence from *in vivo* imaging that cells use reoriented fibers as "train-tracks" to guide their migration away from the primary tumor. The matrix stiffness and collagen density both mediate cellular force generation, with cells exerting greater force on substrates with either increased stiffness or increased collagen density (Kraning-Rush *et al.* 2012).

It was also demonstrated that type I collagen cleavage is a process required for angiogenesis in tumor sites (Seandel *et al.* 2001). Studies performed by our group involving chemical carcinogenesis reaffirm this (Gonçalves *et al.* 2010), since they show the increase in the number of blood vessels and the reduction in the relative volume of collagen in the groups that present more severe prostatic lesions. In this scenario, collagen cleavage enabled the formation of new blood vessels that nourish neoplastic cells favoring tumor progression. These data support the remodeling of the ECM as a fundamental characteristic of reactive stroma in prostate cancer.

On the other hand, some studies affirm benign tumors may have elevated synthesis of collagen and still not alter the distribution of these fibers. Thus, the increase of this component in peripheral areas of the tumor results in the containment of malignant cells, limiting their expansion. However, invasive tumors may acquire the capacity to orchestrate stromal response, leading to collagen restructuring and thus favoring the malignant invasion (Ruiter *et al.* 2002; Tlsty and Coussens 2005).

Like the collagen I network, reticular fibers suffer growing changes with tumor progression. During the pre-invasion process, characterized by Prostatic Intraepithelial Neoplasia (PIN), in rodent prostates, reticular fibers suffer intense fragmentation and degradation, proving to be thicker and assuming a layout perpendicular to the basement membrane (Figure 2c) (Gonçalves *et al.* 2010). However, in spontaneous lesions in older animals as well as in the chemically induced ones of an invasive nature, collagen and reticular fibers appear disperse in the midst of tumor aggregates in close association with transformed epithelial cells, with clear degradation of reticular fibers in the more internal portions of proliferative aggregates (Figure 2c) (Campos *et al.* 2008; Custódio *et al.* 2008; Gonçalves *et al.* 2010). Decreased collagen and reticular fibers were also verified in human prostate tumor stroma (Fávaro *et al.* 2012). Thus the assessment of changes in fibrillar components that affect the stromal environment in prostate cancer may help in the evaluation of the tumor aggressiveness and anaplasia.

Elastic System fibers

Elastic fibers are the least abundant elements of prostatic stroma, and they provide flexibility to the tissue, while acting as a substrate for cell anchorage and migration (Carvalho *et al.* 1997). The prostatic gland of rodents presents few sparse elastic fibers in the glandular stroma, in vessel walls and concentrically arranged to the acini (Carvalho *et al.* 1997). There are, however, only scant data on the role of elastin and its receptors in tumor invasion (Fülöp and Larbi 2002).

Aging and various inflammatory diseases such as atherosclerosis, abdominal aortic aneurysms, chronic obstructive pulmonary diseases, cancer and type 2 diabetes are characterized by the destruction of elastin fibers and the consequent generation of elastin peptides which are biologically active (Fülöp *et al.* 2012). The degradation of the elastin scaffold can result from the action of several enzymes in different tissues, such as neutrophil elastase, pancreatic elastase I as well as metalloproteinases (MMP-2 and MMP-9) (Lapis and Tímár 2002). After degradation, elastin peptides formed induce a variety of biological effects on fibroblasts, phagocytic cells, lymphocytes, smooth muscle cells and endothelial cells, mediated by the elastin-laminin receptor which has been demonstrated to be present on the membrane of these cells (Fülöp *et al.* 2001). Experimental evidence indicates that the 67 kDa elastin-laminin receptor subunit plays an important role in tumor invasion by mediating essential tumor cell functions leading to metastases (Fülöp and Larbi 2002). Elastin and its peptides emerged as possible invasion enhancers for some tumor cells since those molecules are known to stimulate receptor signaling and chemotaxis, which could explain the morphometric changes that have been reported for certain tumor cell lines invading elastic lamina (Parsons, 1993).

In rodent prostates, normal acinar areas revealed elastic fibers arranged in a thin layer under the epithelium, in a close relation with BM (Figure 2e). Tumor sites revealed drastic structural alterations in these elements (Tuxhorn *et al.* 2001; Vilamaior *et al.* 2005). Premalignant lesion, such as PIN, presented the beginning of disorganization of the elastic elements which was accentuated in microinvasive sites. In tumor regions, the change was observed in the pattern of elastic element distribution, which proved to be quite tenuous, fragmented and restricted to the periphery of altered areas (Figure 2d) and around blood vessels that irrigate neoplastic regions. Great scarcity was also observed on foci occupied by microinvasive carcinomas and their reduction as the proliferative focus suffered expansion.

The association of tumor cells with collagen and elastic fibers in the neoplastic region has thus become clearer, revealing the need for more accurate research of the action pathways of these elements during tumor progression (Parsons 1993; Fülöp *et al.* 2012). Nevertheless, much research has to be done in elucidating the exact role of elastin peptides as putative invasion enhancers in tumor stroma and age-related inflammatory diseases (Fülöp *et al.* 2012). This aspect of ECM biology will become an important research field.

BASEMENT MEMBRANE

Basement membrane is a complex of specialized ECM proteins that consists in a layer of 50-100nm in thickness which structurally underlies all epithelia and endothelia (Kalluri 2003; LeBleu *et al.* 2007). A common characteristic is the close relation of this structure with adjacent cells; however its function is not restricted only to guaranteeing mechanical support for the tissue and its compartmentalization, but also to modulate the behavior, the differentiation and the proliferation of cells (Paulsson 1992; Timpl 1996).

The BM is composed by large insoluble molecules that come together to form sheet-like structures in a process known as 'self-assembly', which is driven by cell-surface anchors and receptors (Yurchenco *et al.* 2002). In general all cells are known to produce BM constituents among which the most abundant are type IV collagen, laminin, heparan-sulphate proteoglycans (HSPGs) and nidogen/entactin. Minor components include agrin, SPARC/BM-40/osteopontin, fibulins, type XV collagen and type XVIII collagen (Yurchenco *et al.* 1990; Paulsson 1992; Kalluri 2003).

In order to invade the stroma, carcinoma cells must first breach the BM (Valastyan and Weinberg 2011). In this scenario, type IV collagen seems to be the main BM's structural obstacle, which should be transposed by tumor cells through a proteolytic cascade that acts in its degradation thus permitting tumor invasion (Reich *et al.* 1988; Pezzato *et al.* 2004). Integrin receptors connected to laminins seem to mediate the adherence of tumor cells to basal membrane before and during the invasion. Furthermore, the BM also plays crucial roles in signal transduction events within carcinoma cells via pathways initiated by integrin-mediated cell-matrix adhesions, leading to alterations in cell polarity, proliferation, invasiveness, and survival (Bissell and Hines 2011).

In the current gerbil model of prostate carcinogenesis, the proteolytic degradation of type IV collagen and laminin in malignant sites can be proved by immunohistochemistry (Figure 3a,b) (Gonçalves *et al.* 2010). In these cases, an absence of immunostaining of these elements was observed in some points of neoplastic acini indicating rupture in network formed by these molecules in several glandular regions. Additionally, the ultra-structural analysis of this region revealed a delamination of BM, evidenced by greater distancing between the electron-dense and the electron-lucent layers (Figure 4b). This event is probably related to the loss of association between collagen IV molecules and laminin or degradation of these elements by proteases. The association of various collagen IV molecules forms a network through interactions between the N and C-terminal domains and the laminin associates at less orderly complexes. These two sets of molecules seem to be interconnected, presumably through specific sites thus forming a containment barrier for epithelial cells (Paulsson 1992). Since these molecules are the main constituents of BM its degradation indicates that BM is dissolved and that tumor cells can invade the underlying stroma and metastasize to other organs (Figure 3a,b) (Gonçalves *et al.* 2010; Campos *et al.* 2011). The rupture of BM appears to be a key feature of neoplastic invasion and products generated by this proteolytic process such as collagen IV play an important role during angiogenesis, tissue remodeling and cancer progression (Tanjore and Kalluri 2006).

PROTEOGLYCANS AND ADHESIVE GLYCOPROTEINS

A localized variation in arrangement of stroma macromolecules in response to the prostatic lesions may occur in components other than fibrillar ones (Taboga and Vidal 2003) thus, qualitative and quantitative alterations in glycoprotein components of the ECM affect interactions between the epithelium and stroma (Goulas *et al.* 2000). Among these glycoproteins are proteoglycans that are important modifiers of cellular proliferation and differentiation, playing leading roles in tissue growth and development, in both normal and neoplastic conditions (Yip *et al.* 2006). Many of the functions of proteoglycans are associated with their attached glycosaminoglycan side chains. Chondroitin sulfate is a glycosaminoglycan side chain component of several distinct proteoglycans (e.g., versican) and has a linear polymer structure that possesses repetitive, sulfated disaccharide units containing glucuronic acid and

N-acetylgalactosamine (GalNAc) (Sakko *et al.* 2008). Figure 3e shows the distribution of chondroitin sulfate in areas with proliferative lesions in older gerbils and this seems to play an important role in the initial processes of neoplastic transformation. Acinus with PIN presented a great concentration of glycosaminoglycan among proliferative cells and below areas with normal epithelium (Figure 3e). In microinvasive foci, the expression was practically absent, indicating possible degradation of this component with the progression of the invasion process in the adjacent stroma (Figure 3e). This proteoglycan synthesis turnover was also described in human prostate cancer, with different types being synthesized in the progression to malignant lesions (Kosir and Quinn 1995; Goulas *et al.* 2000).

Tenascin is an ECM glycoprotein expressed predominantly during embryogenesis, wound healing, and neoplastic processes. It has been identified, even as chondroitin sulfate, that tenascin shows multifunctional properties, since this ECM protein works as a modulator of cell-matrix interaction but does not seem to contribute directly to the structural element formation (Murphy-Ullrich 2001; Iyoda and Fukai 2012). Additionally, higher levels of tenascin are related increases in size, degree of severity and tumor stage, that is, aspects that refer to carcinoma aggressiveness (Suwiwat *et al.* 2004).

Tenascin's expression was associated with the appearance of small blood vessels and to the remodeling of the subepithelial stroma in older gerbils with neoplasias (Figure 3c,d). In animals submitted to long periods of steroid blocking, the glycoprotein was sporadically found in the subepithelial region of some acini and associated with blood vessels (Figure 3c,d), but it remained present among proliferative aggregate cells, as occurred in lesions found in animals treated for a short period (not shown). In human prostate lesions, the participation of tenascin in the stromal reaction is more prominent in lesions with a degree of Gleason 3 in relation to Gleason 4 (Tomas *et al.* 2006). Furthermore, newly formed tumor blood vessels and inflammatory and stromal cells take part in the expression of tenascin and are involved in the formation of a provisional tumor matrix. The deposits of tenascin indicate rebuilding processes in non-neoplastic as well as in neoplastic prostatic tissues (Katenkamp *et al.* 2004) and the main cells in prostate cancer stroma responsible for the tenascin production are myofibroblasts (Tomas *et al.* 2006).

Chondroitin sulfate and tenascin have been individually implied in the modulation of cell adhesion and many studies suggest that these matrix elements co-locate in the midst of

neoplastic stroma of tumors. This suggests that such molecules can act jointly to promote anchorage and cell motility (Kosir and Quinn 1995). However, different from the chondroitin sulfate expression, the tenascin expression was evident in all invasive sites, even when these were comprised of a small number of atypical cells. Probably, as this progresses to an invasive process, the glycosaminoglycan degradation rate will increase and new stromal elements will be recruited or synthesized to promote survival, growth and migration of invasive prostatic cells. Our results reinforce that the location of different types of ECM components is altered during the invasive processes.

METALLOPROTEINASES

Neoplastic cells exhibit features, required for local and distant invasion such as their capacity to recognize ECM. Access to growth factors confined to ECM, the onset of angiogenesis and the degradation of ECM elements such as collagen and other glycoproteins, which act as a barrier against tumor invasion, depends on the activation of a complex machinery of proteolytic enzymes (Littlepage *et al.* 2005; Bacac *et al.* 2006). During carcinogenesis elevated levels of proteases, including matrix metalloproteinases (MMPs), are produced by tumor and/or stromal cells which are currently thought to endow cells with invasive properties (Fülöp and Larbi 2002). These MMPs are able to degrade a variety of ECM molecules such as type IV collagen, elastin, laminina (Fülöp and Larbi 2002) and type I collagen, in normal physiological processes and pathological stages (Roy *et al.* 2009; Hatfield *et al.* 2010; Sprenger 2010). They are secreted as pro-enzymes and have to be activated by partial proteolytic cleavage to become biologically active, mainly by plasminogen activator. One important control is exerted by naturally occurring inhibitors such as TIMPs (tissue inhibitors of metalloproteinases) acting at the latent or active forms of MMPs, thus being able to impair tumor cell invasion of the ECM (Fülöp and Larbi 2002).

It is now evident that MMP function is more complex than initially thought, given that these enzymes do more than degrade physical barriers (Kessenbrock *et al.* 2010). Rather, they regulate signaling pathways that control cell growth, survival, invasion, inflammation and angiogenesis (Bauvois 2012). In normal tissue, MMP activity is carefully controlled via transcriptional and post translational mechanisms. Carcinoma cells have designed numerous

means to alter the normally tight control of MMP activity, usually leading to enhanced MMP function. While degrading the BM and other ECM components that lie in the path of invading tumor cells, MMP-expressing cells also liberate growth factors that are sequestered there, thus favoring cancer cell proliferation (Kessenbrock *et al.* 2010). In terms of genetic control, in a recent study was observed that, eHsp90 increases transcript expression of MMP-2, MMP-3, and MMP-9 which favors tumor progression (Hance *et al.* 2012). TGF-β also seems to act in the control of the synthesis of MMPs and their TIMPs (Prud´homme 2007; Kalluri and Han 2008).

MMP-2 and MMP-9 gelatinases are being intensively studied in several animal models (Zhang *et al.* 2004; Roy *et al.* 2009; Bruni-Cardoso *et al.* 2010; Justulin *et al.* 2010) since they participate in the degradation of ECM components, including the BM, and also generates some bioactive fragments originated from the breakdown of ECM components (Hua *et al.* 2011). Our research group has investigated the role of these gelatinases in male and female gerbil prostate after hormonal manipulation (Rochel-Maia *et al.* 2011) and its localization in female prostate during the estrous cycle (Santos *et al.* 2011). We have recently investigated the expression of MMP-2 in prostatic lesions seeking to better characterize the model of induced carcinogenesis proposed. Our experimental model has been presenting a high expression of MMP-2 in lesions induced by carcinogens associated with promoter agents, such as testosterone and hyperlipidic diet. The expression of this metalloproteinase was verified in the epithelial compartment and in the stromal, however, the epithelial cells apparently showed greater immunostaining (Figure 3f). This indicates that the promoters associated with the carcinogen increase tumor cell capacity to degrade ECM elements, through proteolysis by MMPs, and to invade subjacent stroma. The relation between the overexpression for metalloproteinases and tumor progression has been supported by other studies involving rodent models (Egeblad and Werb 2002).

Although cancer cells from various tissues can express members of the MMP and ADAM (a disintegrin and metalloproteinase) families as well as TIMPs, the major source of these proteinases is from stromal cells infiltrating the tumor (Egeblad and Werb 2002). Cancer cells stimulate host cells such as fibroblasts to constitute an important source of MMPs through the secretion of interleukins and growth factors and direct signaling through extracellular MMP inducer (Murphy 2008). The different types of stromal cells produce a

specific set of proteinases and proteinase inhibitors, which are released into the extracellular space and specifically alter the milieu around the tumor (Ardi *et al.* 2007). Therefore, detailed studies especially concerning stromal components, are necessary to better understand the interplay between epithelium and stroma during carcinogenesis.

MULTIPLE FACTORS AND STROMAL CELLS INVOLVED IN EMT

With respect to the prostatic epithelium, cancer cells with stem cell–like properties within the primary tumors are considered major elements in tumor initiation and progression and a possible source of tumor heterogeneity. These cells can derive from transformation of tissue/adult stem cells or from more differentiated cells that acquire stem-like properties (Clevers 2011; Visvader 2011). Recent studies indicate gene profiling divergence in transcriptional programs between cell subpopulations of the prostate cancer (Celià-Terrassa *et al.* 2012). In PC-3 common parental cell line, a cell group showed a tight association between the expression of an epithelial gene program, including E-cadherin (*CDH1*), EpCAM (*TACSTD1*), and desmoplakin (*DSP*), and also genes associated with pluripotency and self-renewal including *KLF4*, *MYC*, *SOX2*, *KLF9*, and *LIN28A* (Liu *et al.* 2011; Gregory *et al.* 2008), and more aggressive attributes of these tumor cells. Another group expressed high levels of many mesenchymal markers (e.g., *VIM*, *SPARC*, and *FN1*) and genes linked to EMT, such as *TWIST2*, *SNAI2*, *ZEB1*, and *RUNX2,* being more invasive through ECM in *in vitro* assays. Additionally, it is important to distinguish between tumor cell subpopulations that have acquired relatively stable mesenchymal-like phenotypes and those subpopulations with strong epithelial phenotypes that can undergo transient EMT. The role of some factors in the control of this gene reprogramming has been evaluated (Albino *et al.* 2012). Among the candidates can be cited the ETS transcription factors and eHsp90. These elements act to activate and repress genes depending on the promoter context (Sharrocks 2001; Yang *et al.* 2004; Seth *et al* 2005) and its downregulation is an additional relevant event in prostate tumorigenesis (Cangemi *et al.* 2008; Kunderfranco *et al.* 2010). The initiation and progression of prostate cancer is also influenced by epigenetic deregulation of gene expression. The histone methyltransferase MMSET (Multiple Myeloma SET domain) is overexpressed in a variety of metastatic tumors in which induces migration, invasion and morphologic alterations in epithelial cells. MMS*Et al*so

alters the expression of genes involved with EMT through the activation of the transcription factor TWIST1 (Ezponda *et al.* 2012). TWIST1 expression promotes invasion and metastasis by the induction of EMT and also provides self-renewal characteristics to neoplastic cells (Yang *et al.* 2010).

Regarding the stroma of the tumoral environment, this "new microenvironment" has been described as 'reactive stroma' because the peritumoral stromal cells demonstrate many features characteristic of wound repair (De Wever and Mareel 2003; Taylor and Risbridger 2008). In this scenario, two cell types have gained prominence in studies concerning about the tumor-stroma crosstalk (Taboga *et al.* 2008). A specific association of fibroblasts with smooth muscle cells (SMC) in distinct compartments of the rat prostate and the phenotypic and functional alterations that they suffer during tumor progression has been demonstrated (Nemeth and Lee 1996).

There is evidence that normal epithelial cells support SMC differentiation, whereas transformed cells lose this capacity, suggesting the existence of paracrine interactions between these types of cell, which are lost during carcinogenesis (Cunha *et al.* 1996). As the tumor grade increased, the SMC become small and atrophic with a reduction in their cytoplasmic content and they gradually lose the interactions between each other. With tumor progression, malignant cells invade the stroma, segregating the SMC (Taboga *et al.* 2008).

Fibroblasts contributing to the tumor stroma have been termed peritumoral fibroblasts, carcinoma-associated fibroblasts (CAFs) or tumor-associated fibroblasts (Kunz-Schughart and Knuechel 2002a,b). These cells do not stimulate the growth of normal epithelial cells under identical conditions, suggesting that CAFs express ECM proteins and growth factors that influence the incipient tumor cells and promote the angiogenesis necessary to maintain epithelial transformation (Bhowmick *et al.* 2004; Dakhova *et al.* 2009; Barron and Rowley 2012). During the malignant transformation of these fibroblasts, they undergo morphological modifications and become cell types with greater capacity for synthesis while also exhibiting notable differences in gene expression profile (Dakhova *et al.* 2009). These cells are able to synthesize collagen I and III, fibronectin, versican, vascular endothelial growth factors (VEGFs) (especially type VEGF-D), SDF 1 and tenascin and also express proteases, fibroblasts activation protein and MMPs (Tomas *et al.* 2006). Part of the synthetic capacity of CAFs is controlled by TGF-β. *In vitro* studies showed that TGF-β1 induced resting fibroblasts

to develop stress fibers and express smooth muscle α-actin (Ronnov-Jessen and Petersen 1993; Peehl and Sellers 1997; 2000; Gerdes *et al.* 2004).

The reactive stroma of the gerbil prostate, as others rodent models and in humans, presents fibroblasts in intense synthetic activity. Ultrastructurally, these are characterized by large amount of synthetic organelles (Figure 4c) and collagen fibrils associated externally. Stromal fibrosis was identified in spontaneous and induced prostatic tumors, becoming part of the reactive microenvironment, at least in the peritumoral region (Gonçalves *et al.* 2010; Campos *et al.* 2011). Additionally, in our model it was common to observe points of close association between the projections of fibroblast extremities and basement membrane, even when it is in the process of delamination (Figure 4a,d). Thus, further research is needed to better characterize these altered stromal cells and understand their role in initiation and progression of prostate carcinogenesis in gerbil.

Experimental studies on the biological mechanisms of prostate cancer have revealed extensive signaling pathways that provide communication between cancer epithelial cells, stromal cells, and the ECM within tumor microenvironments, that are necessary for tumor progression (Corn 2012). Many therapeutic agents have been studied in an attempt to counter these signaling pathways and thus be used for the treatment and even prevention of prostate cancer.

CONCLUSIONS, FUTURE DIRECTIONS AND PERSPECTIVES

Existing data until now indicate that the ECM architecture associated with the tumor (Figure 1) differs greatly from the preexisting stroma and that most of the synthesis, degradation and remodeling of components results from the changes in stromal cell phenotype which characterizes EMT (Tuxhorn *et al.* 2001; Gonçalves *et al.* 2010). Our results indicate that the stromal reaction involves a crosstalk between transformed epithelial cells and activated fibroblasts. The first act in the sense of matrix degradation by increasing the release of proteases such as MMP-2 and the fibroblasts synthesize elements like collagen I, III, tenascin and chondroitin sulfate. Our group has been trying to characterize important steps of prostatic carcinogenesis in Mongolian gerbils since this model has important characteristics in experimental assays such as the development of spontaneous prostatic neoplasia and the

reduction of the tumor latency period in animals initiated by chemical carcinogens. Furthermore, a cell type was recently identified in the stroma of this animal that seems to interact with several other ECM elements as well as with other stromal cells (Telocytes).

COMPETING INTERESTS

The authors declare that they have no conflicts of interest concerning this article.

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FIGURE LEGENDS

Figure 1 Architecture of Extracellular Matrix during prostate cancer progression. During prostate cancer progression epithelial and stromal compartment undergoes several modifications including: ① transformed epithelial cell with ② self-renewal properties ③ loss of cell polarity and acquisition of invasive properties by epithelial cells, ④ phenotypic alterations in stromal cells, ⑤ angiogenesis, ⑥ influx of inflammatory cells to tumor sites, ⑦ extracellular matrix remodeling and ⑧ increased expression of proteases and growth factor. All these alterations endow tumor cells with metastatic characteristcs.

Figure 1.

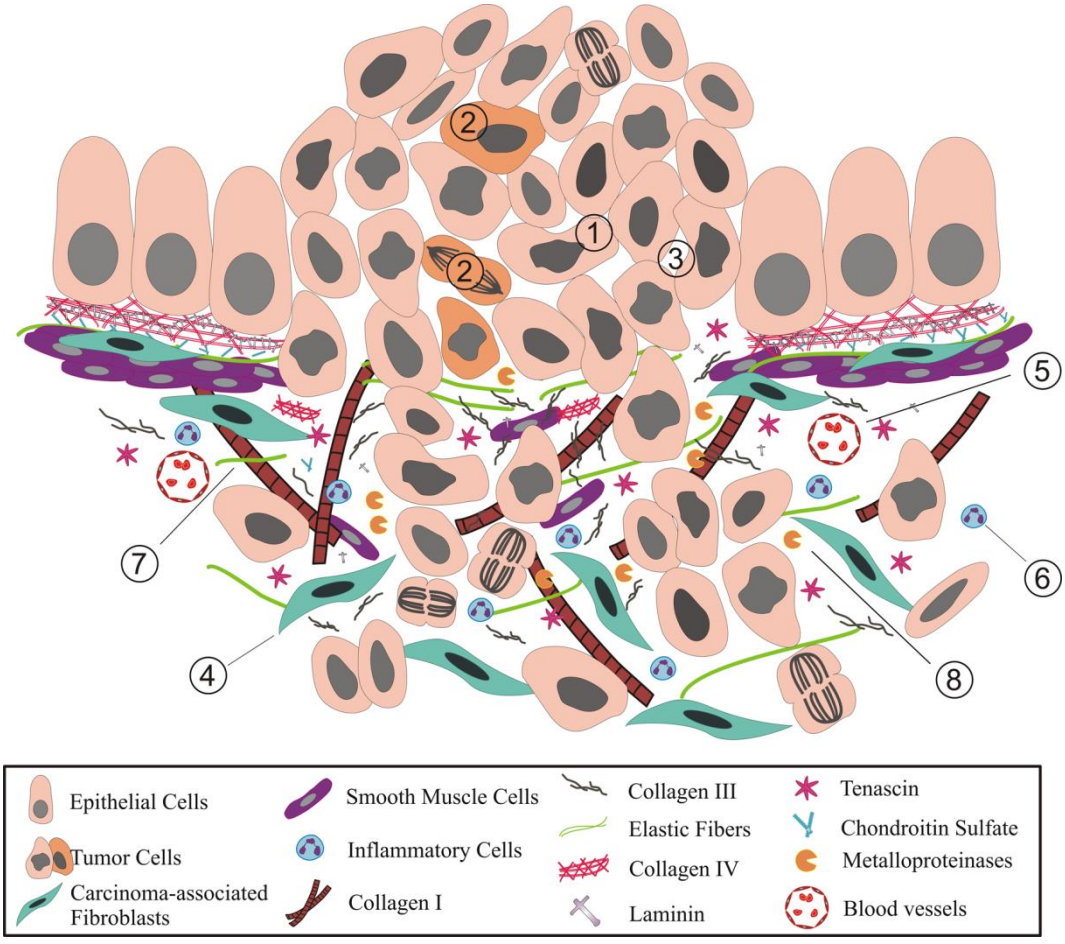

1
2
2
3
4
5
6
7
8
Epithelial Cells
Tumor Cells
Carcinoma-associated Fibroblasts
Smooth Muscle Cells
Inflammatory Cells
Collagen I
Collagen III
Elastic Fibers
Collagen IV
Laminin
Tenascin
Chondroitin Sulfate
Metalloproteinases
Blood vessels

Figure 2 Extracellular matrix fibrillar elements in prostate tumor-stroma. (**A-B**) Collagen type I degradation (black and white arrows) between neoplastic cells in micronvasive carcinoma of gerbil prostate. (**C**) Reticular fibers fragmentation (thin arrows) in proliferative lesions induced by MNU (N-methyl N-nitrosurea) and testosterone. (**D**) Elastic fibers distribution in normal prostate tissue (**E**) Disorganization and reduction of elastic fibers in tumor sites. (A-B) Sirius Red, (B) Polarized light, (C) Gömöri reticulin, (D-E) Fluorescence microscopy of sections stained with hematoxylin eosin. MC: Microinvasive Carcinoma, ep: Epithelium.

Figure 2.

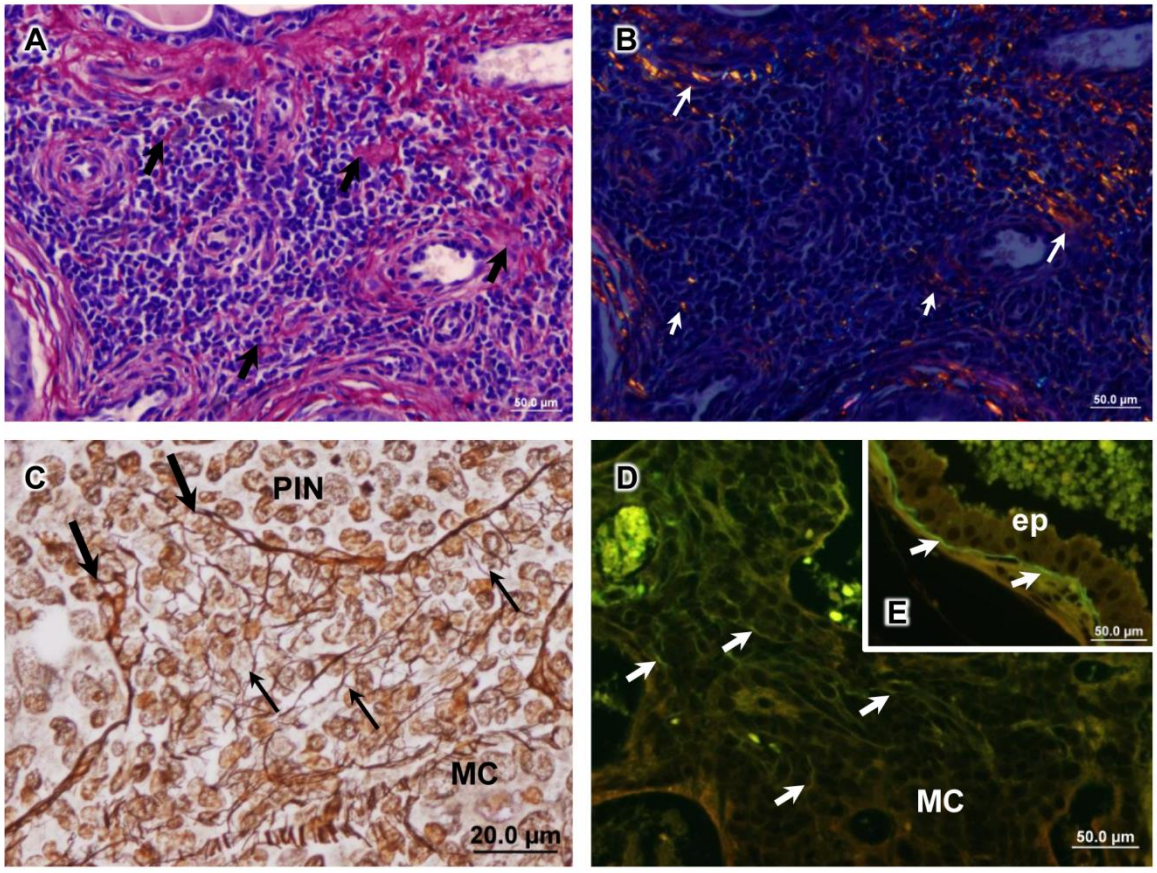


Figure 3 Basement membrane, proteoglycans, adhesive glycoproteins and metalloproteinase expression during prostate carcinogenesis. Rupture of collagen IV (arrow) (**A**) and laminin (arrows) (**B**) network (dashed lines) enabling stromal invasion at neoplastic sites. Positive immunostaining of tenascin in prostate tumor-stroma (**C**) and associated to the emergence of blood vessels (**D**). High concentration of Chondroitin sulfate between proliferative epithelium (arrows) and absence of its expression in microinvasive foci (*) (**E**). MMP-2 high stain in neoplastic epithelium (*) (**F**). MC: Microinvasive Carcinoma, ep: Epithelium.

Figure 3.

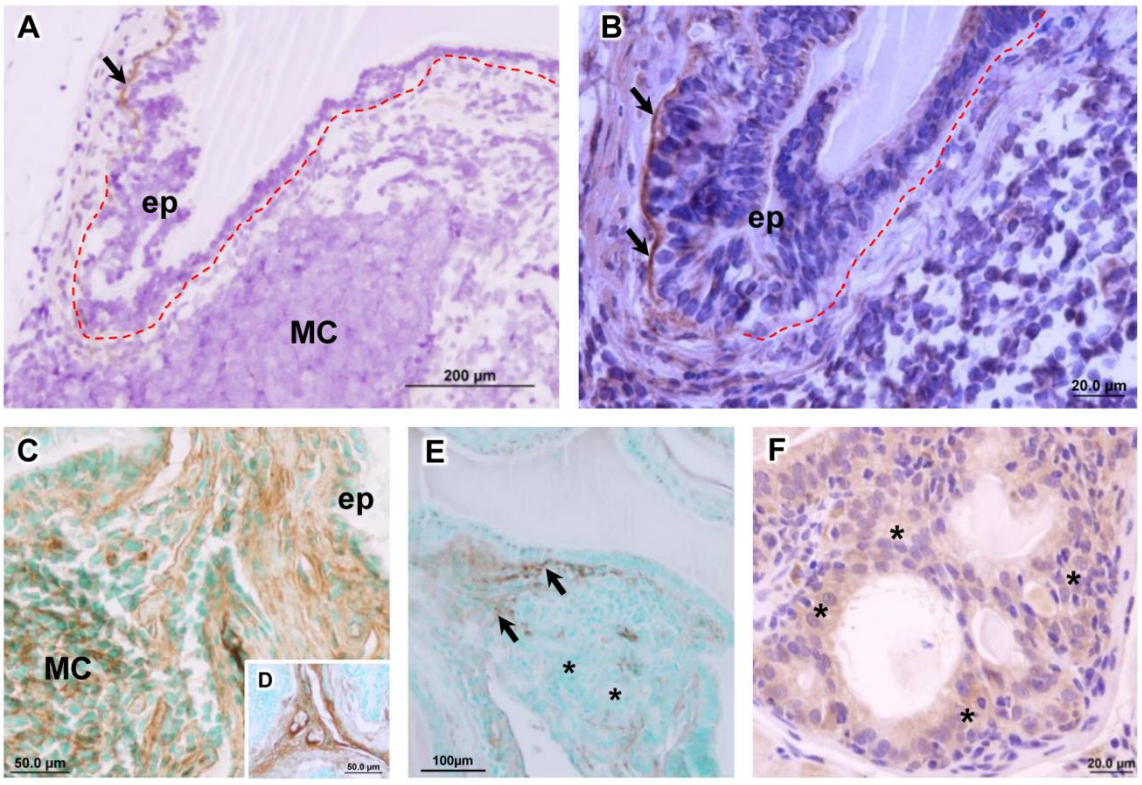
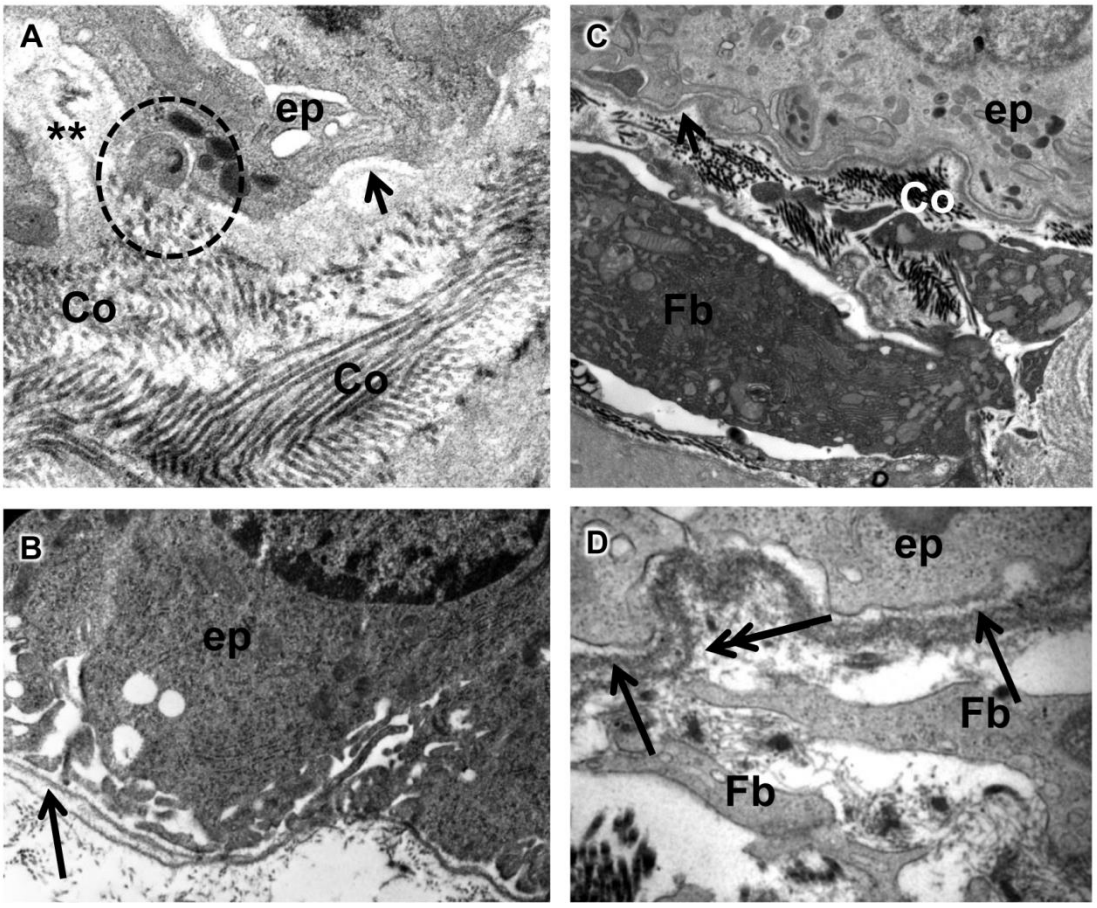


Figure 4 Transmission Electron Microscopy of gerbil prostate. (**A**) Epithelial-stromal interface area where delamination of basement membrane can be observed (**). Abundant Collagen fibers (co) in this region are associated with cells in early migratory process (circle). 27800x. (**B**) Delamination of the basement membrane. 12930x. (**C**) Activated fibroblasts in the subepithelial region. 10000x. (**D**) Association between fibroblast extensions (Fb) and basement membrane (double arrow). 35970x. Ep: epithelium; short arrow: intact basement membrane, long arrow: delamination.

Figure 4.



ANEXOS

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

CERTIFICADO

Certificamos que o projeto de pesquisa "Carcinogênese prostática quimicamente induzida por N-metl-N-nitrosuréia (MNU) em gerbilos da Mongólia: associação com promotores esteroidais ou dieta hiperlipídica" (Protocolo no. 003/2009), sob responsabilidade de Sebastião Roberto Taboga, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela Comissão de Ética em Experimentação Animal (CEEA), em reunião de 27/03/2009.

São José do Rio Preto, 27 de março de 2009

Profa. Dr. Rejane Maira Góes
Presidente - CEEA

Instituto de Biociências, Letras e Ciências Exatas – Departamento de Biologia
Rua Cristóvão Colombo, 2265 CEP 15054-000 São José do Rio Preto – SP – Brasil
Tel 17 221 2391 fax 17 221 2390

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **"Carcinogênese Prostática Quimicamente Induzida Por N-Metil N-Nitrosuréia (MNU) Em Gerbilos Da Mongólia: Associação Com Promotores Esteróides Ou Dieta Hiperlipídica"**:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _____, Instituição: _____.

(X) CEUA - Comissão de Ética no Uso de Animais , projeto nº 003/2009 - CEEA, Instituição: Instituto de Biociências, Letras e Ciências Exatas - UNESP.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _____, Instituição: _____.

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Bianca Facchim Gonçalves
Aluna: Bianca Facchim Gonçalves

Orientador: Prof. Dr. Sebastião Roberto Taboga

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Ana Maria Aparecida Guaraldo

Carimbo e assinatura

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

_____
Carimbo e assinatura